FORD, MARLENA

STEROIDOGENIC ACTIVITY OF THE PLACENTA, CORPUS LUTEUM AND BLOOD OF THE AFRICAN ELEPHANT, *LOXODONTA AFRICANA*

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Steroidogenic activity of the placenta, corpus luteum and blood of the African elephant, *Loxodonta africana.*

by

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ABSTRACT

The study was directed at expanding our understanding of the endocrine correlates of pregnancy in the African elephant (*Loxodonta africana*). This information is considered important for the potential development of techniques to manipulate the reproductive output of female elephants in attempts to control the size of populations in confined areas.

During the gestation period no obvious trends for the concentrations of progesterone and 5α-dihydroprogesterone could be illustrated. The concentrations of both progestins were higher in pregnant than in non-pregnant females. Circulating concentrations of 5α-dihydroprogesterone were higher than that of progesterone in pregnant animals, but not in non-pregnant females or foetuses. Concentrations of oestradiol-17β were low in all the samples tested.

Pregnenolone was converted to polar compounds by placenta. Progesterone was the principal metabolite in blood. Corpora lutea were the main sites for the synthesis of 5α-reduced metabolites and therefore provide the most suitable site for possible control.

ACKNOWLEDGEMENTS

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CHAPTER 1

Introduction

Elephants in South Africa

Elephants in South Africa have increased from about 120 individuals in 1920 to more than 8 500 in 1992 (Hall-Martin 1993). At present most of these animals are confined to nature reserves where they are protected from poaching and harassment. As a result these populations are growing. The mean rate of population increase of the elephant population in the Kruger National Park in the past 30 years, from 1967 to 1997, has been estimated at 5% per year (Whyte, Van Aarde & Pimm 1998). This approximates the theoretical maximum rate of increase for elephant populations, calculated at 7% per year by Calef (1988). Elephants can have a dramatic impact on their environment and consequently influence the species-composition of that environment (Dublin 1994; Leuthold 1996). In an attempt to avoid the possible loss of species diversity or the loss of vulnerable species (Höft & Höft 1995) the elephant population in the Kruger National Park is maintained through culling at approximately 7 500 individuals (Smuts 1975). The culling programme was initiated in 1968 (Smuts 1975) and was later (1982) supplemented by the translocation of some juveniles (Bengis 1996). Such a management approach creates ethical and logistical problems.

As an alternative management option, the size of elephant populations in confined areas may be controlled by limiting their reproductive output. Stages of the female reproductive cycle which may be artificially manipulated to control reproductive output, include gametogenesis, fertilisation,

implantation (before or after), age at first conception, inter-calving interval and age at reproductive senescence (Heap 1972). Whichever method is selected, interference with the social structure and status of the target species should be minimised. In social species, such as the African elephant, the removal of conceptuses after implantation and the unnatural introduction of reproductive senescence are therefore unacceptable.

The age of sexual maturity of females and the mean calving interval are important demographic parameters that influence the rate of population growth (Calef 1988). Attempts to limit the growth rate of elephant populations should therefore focus on methods to increase the age at sexual maturity of females and lengthen the calving interval. Interference with reproductive output can also be attempted at an endocrine level. The production of reproductive hormones may be manipulated at their sites of synthesis, through the possible inhibition of metabolic enzymes or the removal of hormone precursors. The sites of synthesis are the corpora lutea and possibly the placenta and blood. Hormones are often bound to plasma proteins for transport to their target tissues (Pardrige 1988) and this may be another possible site for manipulation. Finally, the action of the reproductive steroids at the target tissues may be manipulated through natural and synthetic steroids that bind to target receptors. However, the control of the reproductive output of female elephants has to be based on a thorough knowledge of endocrine and behavioural aspects that underlie reproduction in the species.

Reproductive parameters of female elephants

Elephant females reach puberty by the age of 9-22 years. Pregnancy lasts 22 months and cows lactate for 2-3 years. The inter-calving interval varies from 3 to 9 years and is influenced by environmental conditions, especially rain, as well as the density of the population (Perry 1953; Laws 1969). Two types of oestrous cycles have been described by Kapustin, Critser, Olson & Malven (1996). A short nonluteal cycle (2.9 weeks) is alternated with a longer (10.6 week) luteal-active cycle to give a combined total oestrous cycle length of 13.5 weeks.

Pregnancy in this species is not associated with elevated concentrations of plasma progesterone, as is the case with most other mammals (Heap 1972). Early studies on plasma progesterone levels reported no (Hanks & Short 1972) or low levels (Plotka, Seal, Schobert & Schmoller 1975) and an overlap in the concentrations measured in plasma from pregnant and non-pregnant females (McNeilly, Martin, Hodges & Smuts 1983; De Villiers, Skinner & Hall-Martin 1989). Short & Buss (1965) and Short (1966) could not measure any progesterone in elephant corpora lutea. Although Smith, Hanks & Short (1969) claimed that the corpora lutea of African elephants do not produce progesterone, Hodges, Van Aarde, Heistermann & Hoppen (1994) showed that corpora lutea do have the ability to convert pregnenolone to progesterone, 5α-pregnane-3,20-dione (trivial name: 5α-dihydroprogesterone) and 5α-pregnane-3α-ol-20-one. The biosynthesis of these 5α-reduced forms of progesterone in the corpora lutea (Hodges *et al.* 1994) and their concentrations in the plasma, which both exceeds that of progesterone, is apparently a better reflection of ovarian function than progesterone (Hodges, Heistermann, Beard & Van Aarde 1997). This lead to the suggestion that these 5α-reduced forms are indeed the biologically active progestins in the African elephant. More

recently it has been shown that uterine progesterone receptors has a high (43% for 5α-dihydroprogesterone and 20% for 5α-pregnane-3α-ol-20-one) relative binding affinity for these 5α-reduced progestins (Greyling, Van Aarde & Potgieter 1997). Furthermore, 5α-dihydroprogesterone down-regulates the uterine progesterone receptor (Greyling, Ford, Potgieter & Van Aarde 1998), which confirms the biological significance of this 5α-reduced metabolite.

Contraception

The ideal method to manipulate the fertility of free-ranging animals should not only be effective in lowering the reproductive output of females, but it should also have minimal effects on the behaviour and health of the animals. The treatment should also be reversible and safe for use in non-pregnant, pregnant and lactating females (Turner, Liu & Kirkpartick 1996). The contraceptive chemicals has to be metabolised in the bodies of the animals to such an extent that they do not remain in the food chain (Turner & Kirkpatrick 1991). Another requirement is remote delivery of the contraceptive. Steroid contraceptives, although successful in limiting the reproductive output of females, may require large doses or frequent injections to maintain the levels necessary to be effective (Turner *et al.* 1996). This is especially true for natural steroids. Furthermore, the natural steroids have the advantage of being metabolised in the body. Synthetic steroids may have many side-effects, including those on behaviour and ovarian, uterine and mammary tissue (e.g. Li, Li, Klicka, Parsons & Lam 1983; Yager, Campbell, Longnecker, Roebuck & Benoit 1984; Friedman, Lobel, Rein & Barbieri 1990; Turner & Kirkpatrick 1991; Turner *et al.* 1996).

An alternative to steroid-induced fertility control is immunocontraception by the porcine zona pellucida vaccine (Turner & Kirkpartick 1991). The porcine zona pellucida vaccine contains an antibody against a zona glycoprotein (ZP3), which prevents fertilisation by blocking sperm-zona interactions (Sacco, Subramanian & Yurewicz 1984; Florman & Wassarman 1985). Due to the protein nature of this contraceptive treatment, it is readily metabolised and therefore cannot accumulate in the tissues of predators or scavengers. It apparently also does not disrupt existing pregnancies (Turner & Kirkpartick 1991) and is reversible in feral equids (*Equus asinus*, Turner *et al.* 1996). However, side-effects of this treatment, including ovarian malfunction, altered cyclicity, abnormal follicular differentiation, reduced follicular development (Liu, Bernoco & Feldman 1989), the lack of folliculogenesis and the depletion of primordial follicles (Paterson, Wilson, Van Duin & Aitken 1996) has been observed in some species. The similarity of the zona pellucida glycoproteins between species permits cross-reactivity of the antibody and it has been shown that those raised from pigs do cross-react with elephant zona pellucida (Bertschinger, Fayrer-Hosken, Kirkpartick, Soley, Steffens & Ard 1996). The potential success of this method in the African elephant is being assessed at present in the Kruger National Park.

The elephant research programme

The present study is part of a programme that aims at finding and/or developing alternative techniques to control the growth of elephant populations deemed to have a negative influence on the maintenance of biological diversity in areas to which they are confined. These alternative methods will focus on the manipulation of the reproductive output of female elephants. Before such a programme can be attempted, it is vital to be familiar with the endocrine parameters that influence

and control reproduction in the species. Initial research lead to the proposal that 5α-reduced progestins were of biological significance in elephant females (Hodges *et al.* 1994). In the present study the circulating concentrations of one of these 5α-reduced progestins, namely 5α-dihydroprogesterone, as well as that of progesterone itself, will be determined in the plasma collected from female elephants in different reproductive stages, in order to determine the relative biological importance thereof during various stages of the reproductive cycle. Hodges *et al.* (1994) illustrated that the corpora lutea of African elephant females have the ability to produce progesterone, 5α-dihydroprogesterone and 5α-pregnane-3α-ol-20-one from pregnenolone. In many other mammals the corpora lutea are the main structures responsible for the production of progesterone (Baird 1984; Miller 1988). The steroidogenic potential of the other sites of progestin-synthesis, namely the placenta (e.g. Strauss, Martinez & Kiriakidou 1996) and blood (e.g. Van der Molen & Groen 1968) will be investigated here. The present study will therefore add to our knowledge of the sources and circulating concentrations of progestins during pregnancy. Other facets of the research programme include the characterisation of the uterine receptors (Greyling 1997) and plasma binding proteins for these progestins. The structures responsible for the synthesis, transport and biological action of progestins all provide possible sites for the manipulation of the reproductive process in the female African elephant.

Aims of the present study

The aims of the present study are to determine the relationship between the plasma concentrations of reproductive hormones (progesterone, 5α-dihydroprogesterone and oestradiol-17β) and the reproductive status of female African elephants and to determine the potential of whole blood

(cellular and plasma components separately), placenta and corpora lutea to produce progestins through the metabolism of pregnenolone.

The specific objectives of the study are as follows:

- to determine whether circulating concentrations of progesterone, 5α-dihydroprogesterone and oestradiol-17β are indicative of the reproductive status of female African elephants and
- to determine the potential of elephant placenta, corpus luteum and blood to metabolise pregnenolone (a precursor of steroid hormone synthesis).

CHAPTER 2

Circulating concentrations of progesterone, 5α-dihydroprogesterone and oestradiol-17β in female African elephants.

Introduction

Research over the last two decades on reproduction in the female African elephant focused on hormonal correlates of the oestrous cycle (e.g. Brannian, Griffin, Papkoff & Terranova 1988; Plotka, Seal, Zarembka, Simmons, Teare, Phillips, Hinshaw & Wood 1988; Kapustin *et al.* 1996; Wasser,Papageorge, Foley & Brown 1996) and of pregnancy (e.g. Plotka *et al.* 1975; Hodges, Henderson & McNeilly 1983; De Villiers *et al.* 1989; Hodges *et al.* 1994; Hodges *et al.* 1997 and references therein). Much effort has also been devoted to finding reliable indicators of the reproductive status of female elephants (e.g. McNeilly *et al.* 1983; Hodges *et al.* 1983; Niemuller, Shaw & Hodges 1993; Olsen, Chen, Boules, Morris & Coville 1994; Trohorsch, Hodges & Heistermann 1996). The failure of some of these efforts can be accounted for by the fact that pregnancy in the elephant is associated with very low concentrations of progesterone in peripheral plasma (Hanks & Short 1972; Plotka *et al.* 1975; McNeilly *et al.* 1983; De Villiers *et al.* 1989). Furthermore, Short & Buss (1965) and Short (1966) noted that elephant corpora lutea did not contain measurable amounts of progesterone and Smith *et al.* (1969) claimed that the elephant corpus luteum could not produce progesterone. However, Hodges *et al.* (1994) showed that elephant luteal tissue do have the ability to convert pregnenolone to progesterone, 5α-pregnane-3α-

ol-20-one and 5α-dihydroprogesterone. Recently Greyling *et al.* (1997) demonstrated that the endometrial progesterone receptor of the elephant has an affinity for all these progestins. Hodges *et al.* (1997) proposed that circulating levels of 5α-reduced metabolites of progesterone reflects better on luteal function than the levels of progesterone do. Thus, it follows that 5α-reduced metabolites may be of functional importance in maintaining pregnancy in this species. Since luteal concentrations of 5α-dihydroprogesterone in the African elephant exceed those of progesterone by two orders of magnitude (Hodges *et al.* 1997) it is not surprising that plasma concentrations of 5α-dihydroprogesterone also exceed those of progesterone (Hodges *et al.* 1997). These plasma concentrations may therefore be a better indicator of reproductive status than progesterone and it has been suggested that the circulating concentrations of 5α-dihydroprogesterone are negatively correlated to gestational age (Hodges *et al.* 1997). However, this suggestion is based on samples from 30 elephants (21 pregnant and 9 non-pregnant cows). In the present chapter the validity of this suggestion of defined trends in progestin concentration with gestation will be evaluated for 73 cows (for 5α-dihydroprogesterone and 65 for progesterone) of known gestational stage and 28 non-pregnant cows.

Materials and methods

Collection of material

Material was obtained during the population control programme in the Kruger National Park from 1993 to 1995. Blood was collected by exsanguination into heparinised glass tubes and stored on ice for transport to the laboratory, where it was centrifuged and the plasma removed. All plasma samples were stored at -20°C until the assays were done.

Foetuses were weighed in the field and the mass used in the growth formula of Craig (1984) to determine foetal age and stage of gestation for pregnant females. Pregnant females were placed into one of three categories. Females carrying foetuses too small to weigh up to those that had foetuses aged seven months, were considered to be in early pregnancy. Females with foetuses between, and including, eight and 15 months were categorised as mid-pregnant and females with foetuses from 16 to 22 months old were considered as late pregnant. Ovaries were inspected for the presence of luteal bodies and non-pregnant females were classed as being either sub-adult (no ovarian activity) or lactating (developed mammary glands and milk could be expressed).

Determination of progesterone levels

Extraction

Duplicate aliquots of 100µl plasma were extracted with 4 ml of analytical grade petroleum ether (distillation range: 40-60°C from Saarchem, Krugersdorp, S.A.) by vortexing for 5 min (IKA-Vibrax, Janke & Kunkel, Straufen, Germany) followed by freezing at -20°C for 60 min. and at -70°C for 5-10 min. The organic phase was decanted and evaporated under a stream of N_2 at 37°C. This dried residue was reconstituted in 100µl phosphate buffered saline (PBS, 0.1% gelatin, pH 6.8-7).

Hormone assays

Concentrations of circulating progesterone were determined in a radioimmunoassay (RIA) similar to that described in Van Aarde (1985). A series of standards ranging from 78 to 10 000 pg/ml was

prepared by dilution from a stock solution of 2mg of progesterone (Δ-pregnene-3,20-dione, Sigma, St. Louis, U.S.A.) per 100 ml of absolute ethanol (Saarchem, Krugersdorp, S.A.). Antibody (100µl of a 1:1 500 dilution) was added to 100µl of each of the standards or plasma extracts and the tubes shaken for 1 min. on a Multitube vortexer (Scientific Manufacturing Industries, Model 2601, Emeryville, U.S.A.). The contents of the tubes were incubated for 10 min. at room temperature after which 100µl tritiated progesterone ([1,2,6,7- ^{3}H] progesterone, specific activity: 92 Ci/mmol, ca. 20 000 dpm, Amersham, Buckinghamshire, U.K.) was added. The tubes were shaken for 1 min. and incubated at 4°C for 12-24 h. Unbound steroid was removed with 0.16% dextran coated charcoal (750µl per tube, charcoal from E. Merck, Darmstadt, Germany, Dextran T70 from Pharmacia, Uppsala, Sweden), which was incubated for 13 min. at 4°C followed by centrifugational separation (Hitachi 50PR-22 refrigerated centrifuge, PNI Scientific, Johannesburg, S.A.) at 2 500 rpm for 15 min., also at 4°C. The supernatant was decanted into scintillation vials (Pony Vial, Packard, Downers Grove, U.S.A.) and 4 ml of scintillation cocktail (Ultima Gold XR, Packard, Downers Grove, U.S.A.) was added. The radioactivity was determined after 3-4 h in a Packard 1 500 liquid scintillation counter set to count each vial for 2 min. Hormone concentrations were calculated through interpolation using software (SecuRia 2 000) purchased from Packard Instruments (Packard, Downers Grove, U.S.A.). Buffer (PBS) blanks were included in each assay.

Determination of levels of 5α-dihydroprogesterone

Extraction

Duplicate plasma samples (50µl, 100µl and 200µl, depending on reproductive status) were extracted with diethyl ether (Rectapur, Prolabo, Fontenay-sous-Bois, France) by vortexing for 5

min. (Multitube vortexer, Scientific Manufacturing Industries, Model 2601, Emeryville, U.S.A.) followed by freezing at -20°C for 60 min. and at -80°C for 10 min. The unfrozen ether phase was decanted and evaporated under a stream of N_2 at 37°C. The dried residue was reconstituted in assay buffer (PBS, pH 7 - 7.5).

Hormone assays

The levels of 5α-dihydroprogesterone in the plasma extracts were determined in an amplified enzyme-linked immunoassay (AELIA) similar to the method described by Hamon, Clarke, Houghton, Fowden, Silver, Rossdale, Ousey & Heap (1991). A series of standards ranging from 0 to 30 000 pg/ml was prepared by dilution from a stock solution of 1mg of 5α-dihydroprogesterone (Sigma, St. Louis, U.S.A.) per 1ml of absolute ethanol. The wells of an assay plate (Nunc-Immuno Plate, Nunc, Denmark) were coated at 4°C overnight with 100µl of antigen (11α-hydroxy-dihydroprogesterone conjugated to bovine serum albumin, 0.5µg/ml) diluted 1:2 000 with PBS. The plates were washed four times with 0.05% Tween-20 (Sigma, St. Louis, U.S.A.), after which 50µl of standard or reconstituted plasma extract was added to the wells. The antiserum was diluted 1:400 in PBS and 50µl were added to each well. The plates were incubated at 4°C for 1-2 h, after which they were washed and 100µl of conjugate (goat anti-rat immunoglobulin G labeled with alkaline phosphatase, Sigma Immuno Chemicals, St. Louis, U.S.A.), diluted 1:10 000, was added to each well. After an incubation of 20 min. at 4°C the plates were washed again. NADPH, which served as a substrate for the alkaline phosphatase, was added (100µl per well). After a further incubation of 10 min. at room temperature, amplifier (diaphorase and alcohol dehydrogenase) for the reaction was added to each well and the colour development was terminated by the addition of

100µl of 0.3M of H_2SO_4, after approximately 7 min. The optical density was measured at 490nm in a V_{max} kinetic microplate reader (Molecular Devices, Novo BioLabs, Cambridge, U.K) against an assay buffer blank.

Determination of levels of oestradiol-17β

Extraction

Duplicate aliquots of 200µl plasma were extracted in the same manner as described for the progesterone extraction, except that 3ml of diethyl ether (analytical grade, Saarchem, Krugersdorp, S. A.) were used.

Hormone assays

Circulating levels of oestradiol-17ß were determined from plasma extracts in a similar RIA as that described for progesterone, except that there was no incubation period after the addition of the antibody and the labeled steroid used was [2,4,6,7-^{3}H] oestradiol with a specific activity of 75Ci/mmol (Amersham, Buckinghamshire, U.K.). The series of standards ranged from 156 to 20 000 pg/ml and was prepared by dilution of 2mg of oestradiol (1,3,5[10]-estratriene-3,17β-diol, Sigma, St. Louis, U.S.A.) per 100 ml of absolute ethanol.

Statistical analyses

All statistical procedures followed Zar (1984). Means are followed by one standard error of the mean (s.e.m.). The relationship between the concentration added and the concentration measured in the assay was determined by least squares linear regression analysis and the slope of the line for each

hormone was compared to a line with a slope of one by means of a *t*-test (Fowler & Cohen 1990). Similarly, for the determination of parallelism, the percentage binding was plotted against the concentration of the hormone (both for standards and for serially diluted plasma), the lines fitted using least squares linear regression and the slopes of the two lines compared by means of a *t*-test. Hormone concentrations were tested for homogeneity with the Cochran C, Hartley F-max and Bartlett Chi-square tests for homogeneity of variances. The concentrations of none of the hormones were homogeneous, therefore the Kruskal-Wallis *H* and Mann-Whitney *U* tests were used to determine differences in steroid concentrations between the different groups. Significance was taken at the 95% level.

Validation

Removal of steroids from plasma

Steroid hormones were removed from a pool of plasma with 1% dextran-coated charcoal. Equal volumes (2 ml) of plasma and dextran-coated charcoal were mixed for 10 min. and then centrifuged for 15 min. at 2 500 rpm. The supernatant (stripped plasma) was removed and stored at -20°C for use in the validation of the radioimmunoassays.

Progesterone

To determine parallelism, duplicate aliquots of four volumes of plasma (25µl and 50µl, each made up to a volume of 100µl with PBS, 100µl and 200µl plasma) were assayed as described for the plasma samples. For further validation of the progesterone assay for elephant plasma, duplicate

aliquots (100µl, 50µl and 25µl respectively) of progesterone in PBS (5 000 pg/ml) were added to three volumes (100µl, 150µl and 175µl respectively) of charcoal-stripped elephant plasma. These were assayed as described for the plasma samples to determine the recovery of progesterone from the assay.

The progesterone antibody (1529), supplied by Prof. R.P. Millar of the University of Cape Town, S.A., was raised in a goat against progesterone-11-succinyl-bovine serum albumin. Cross-reactions with other steroids, as determined by the supplier, were as follows: 11α-hydroxyprogesterone: 85%, 17α-hydroxyprogesterone: 12.5%, 5β-pregnane-3,20-dione: 12.5%, 5α-pregnane-3,20-dione: 3%, 5β-pregnane-3β-ol-20-one: 1.73%, 11-deoxycorticosterone: 1.1%, 5α-pregnane-3β-ol-20-one: 1%, 20α-hydroxypregn-4-ene-3-one, 20β-hydroxypregn-4-ene-3-one, 11-deoxycortisol, testosterone, androstenedione, pregnenolone, 5β-pregnane-3α,20α-diol and oestradiol-17β: < 0.7%. Intra- and inter-assay coefficients of variation were 5.8% (n = 6) and 13.5% (n = 6) respectively and the mean recovery of progesterone (1 250 pg/ml) added to stripped plasma was 93 ± 5.2% (n = 4). The slope (b = 0.997) of the line relating the concentrations of progesterone measured to the concentration of progesterone added to stripped plasma (Fig. 1a) were not significantly different from 1 (t_7 = 0.0006). The mean recovery of ^{3}H-progesterone extracted from plasma was 75.0 ± 0.9% (n = 11). Final concentrations were corrected for the extraction efficiency. The slope of the line relating percentage binding and serial volumes of plasma (Fig.1b) was parallel to that of the standard curve (t_5 = 0.13). The detection limit of the assay, defined as two standard deviations of the buffer blank, ranged from 5 to 30 pg/ml.

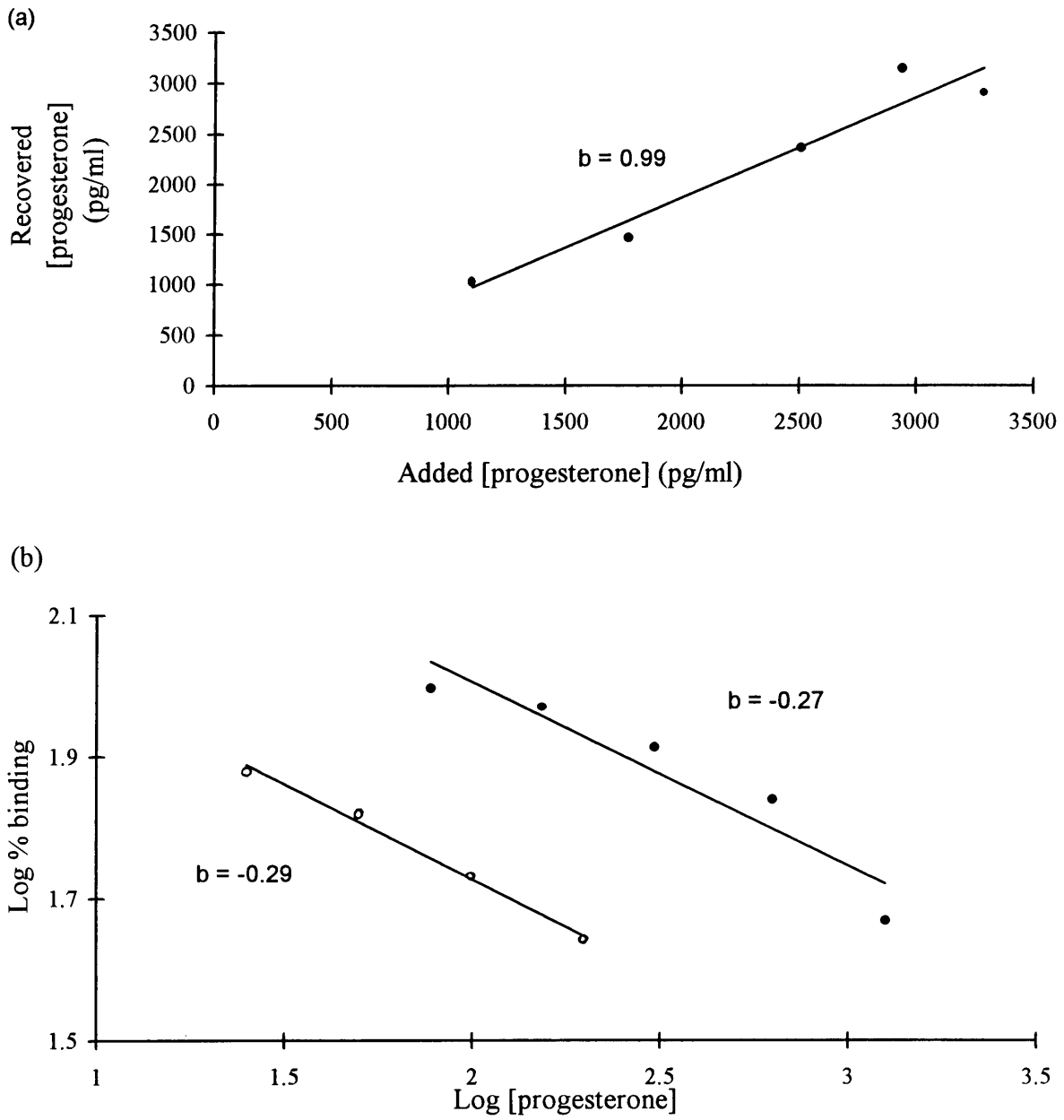

Figure 1 (a) The relationship between the concentrations of progesterone added to stripped plasma and the concentrations of progesterone measured in the assay. The lines were fitted using least squares linear regression and b represents the slope of the line. (b) The slope of the displacement curve of progesterone concentrations of serially diluted elephant plasma (∘ b = -0.29) were parallel ($t_5 = 0.13, p > 0.05$) to that of the progesterone standards (• b = -0.27).

5α-dihydroprogesterone

To determine parallelism, duplicate aliquots of three volumes of plasma (25µl and 50µl, each made up to a volume of 100µl with PBS, and 100µl plasma) were assayed as described for the plasma samples. For further validation of the 5α-dihydroprogesterone assay for elephant plasma, duplicate aliquots (100µl) of 5α-dihydroprogesterone (10 000 pg/ml and 30 000 pg/ml) were added to an equal volume of stripped elephant plasma. These were assayed as described for the plasma samples.

The monoclonal antiserum, raised in a rat, was supplied by M. Hamon of the Babraham Institute, Cambridge, U.K. Cross-reactivities of the antibody, described by Hamon *et al.* (1991), were as follows: 5α-dihydroprogesterone: 100%, progesterone: 56%, 5α-pregnane-3β-hydroxy-20-one: 21%, 5β-dihydroprogesterone: 17%, pregnenolone: 5%, 20α-dihydroprogesterone: 1.2%, 20β-dihydroprogesterone, 5α-pregnane-20α-hydroxy-3-one, 5α-pregnane-3β,20α-diol, Δ^5-pregnene-3β,20α-diol, Δ^5-pregnane-3β,20β-diol, equilin and equilenin: < 0.01%. Intra- and inter-assay coefficients of variation were 9.4% (n = 4) and 16.8% (n = 4) respectively and the mean recovery of 5α-dihydroprogesterone (5 000 pg/ml) added to stripped plasma was 76.0 ± 4.0% (n = 6). The slope (b = 0.72) of the line relating the concentrations of 5α-dihydroprogesterone measured to the concentration of 5α-dihydroprogesterone added to stripped plasma (Fig. 2a) was not significantly different from one (t_6 = 0.55). The slope of the line relating optical density and serial volumes of plasma (Fig. 2b) was parallel to that of the standard curve (t_3 = 0.10). The limit of detection, determined as described for progesterone, of the assays ranged form 50 to 90 pg/ml.

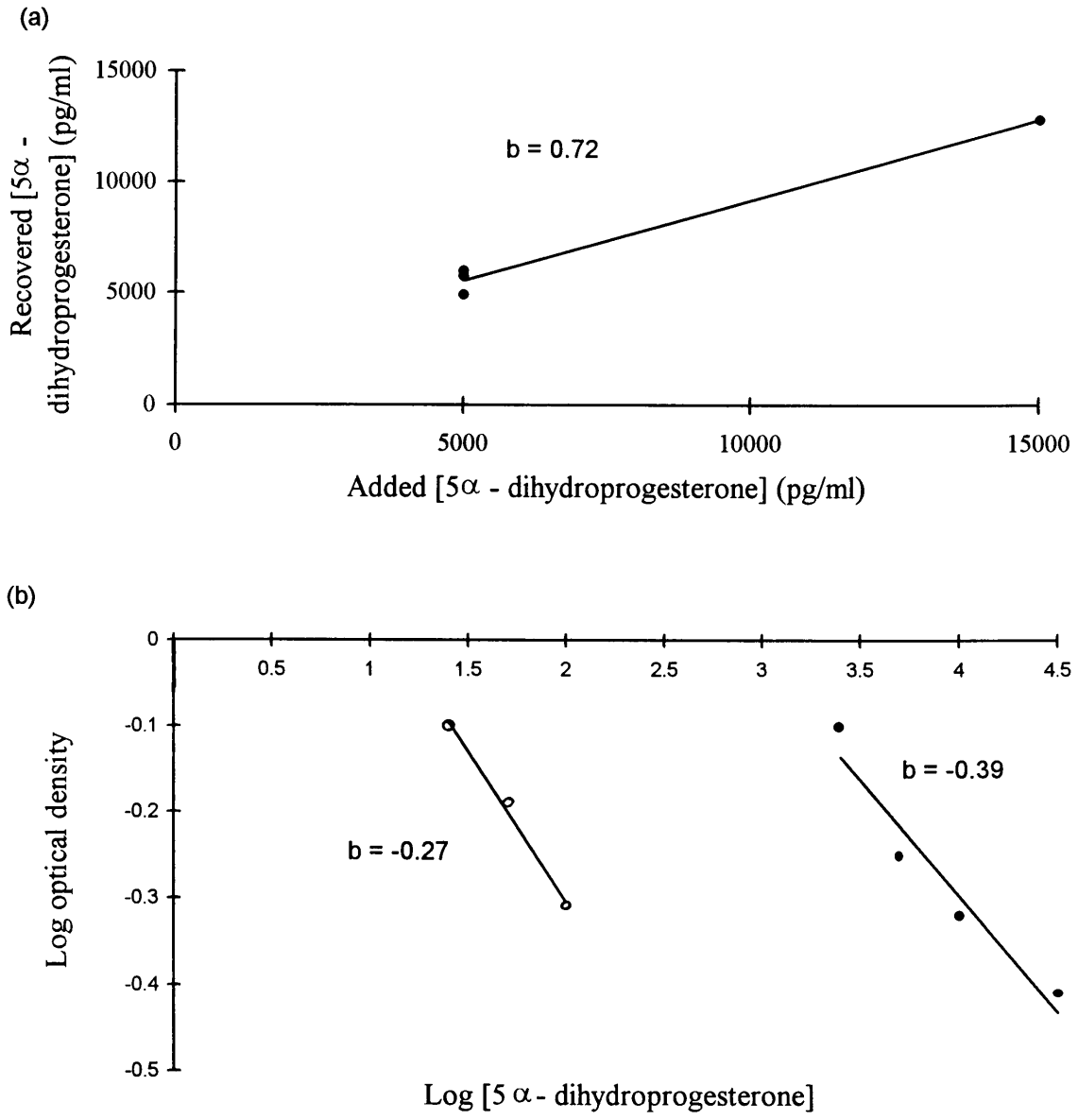

Figure 2 (a) The relationship between the concentrations of 5 α-dihydroprogesterone added to stripped plasma and the concentrations of 5 α-dihydroprogesterone measured in the assay. The lines were fitted using least squares linear regression and b represents the slope of the line. (b) The displacement curve of 5 α -dihydroprogesterone concentrations of serially diluted elephant plasma (• b = -0.39) was parallel ($t_3 = 0.1), p > 0.05$) to that of the 5 α-dihydroprogesterone standards (∘ b = -0.27).

Oestradiol-17β

To determine parallelism, duplicate aliquots of four volumes of plasma (25µl and 50µl, each made up to a volume of 100µl with PBS, 100µl and 200µl plasma) were assayed as described for the samples. For further validation of the oestradiol-17β assay for elephant plasma, duplicate aliquots (100µl) of oestradiol-17β (20 000 pg/ml, 10 000 pg/ml, 5 000 pg/ml, 2 500 pg/ml and 1 250 pg/ml) were added to an equal volume of charcoal-stripped elephant plasma. These were assayed as described for the plasma samples.

The antibody (E29BI) supplied by Prof. R.P. Millar of the University of Cape Town, S.A., was raised in a rabbit against a conjugate of oestradiol-6-(O-carboxymethyl) oxime:bovine serum albumin. Cross-reactivity with other steroids, as described by the supplier, was as follows: 17β-oestradiol: 100%, oestrone: 0.01%, cortisol: 0.005%, deoxycorticosterone: 0.002%, corticosterone: 0.001%, 17α-hydroxypregnanolone, androstenedione, progesterone and testosterone < 0.001%. Inter- and intra-assay coefficients of variation were 5.2% (n = 4) and 5.3% (n = 4) respectively and the mean recovery of oestradiol-17β (2 500 pg/ml) added to stripped plasma was 92.5 ± 1.5% (n = 6). The slope (b = 0.98) of the line relating the concentrations of oestradiol-17β measured to the concentration of oestradiol-17β added to stripped plasma (Fig. 3a) were not significantly different from one (t_7 = 2.37). The mean recovery of ^{3}H-oestradiol extracted from plasma was 90.0 ± 2.1% (n = 18). The final concentrations were corrected for this extraction efficiency. The slope of the line relating percentage binding and serial volumes of plasma (Fig. 3b) was parallel to that of the standard curve (t_5 = 2.25). The detection limit of the assays, determined as described for progesterone, ranged from 0.51 to 3.90 pg/ml.

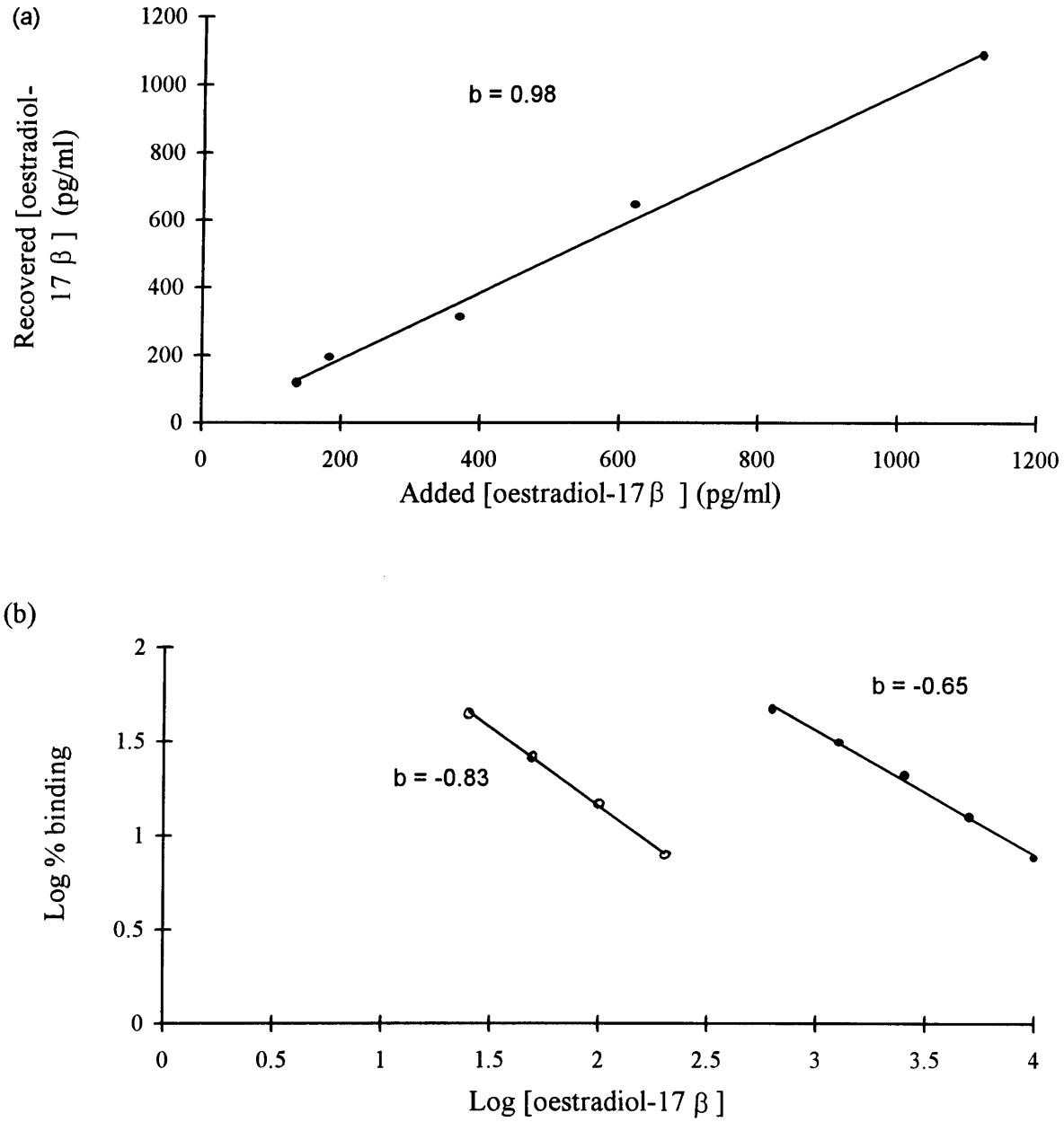

Figure 3 (a) The relationship between the concentrations of oestradiol-17β added to stripped plasma and the concentrations of oestradiol-17 β measured in the assay. The lines were fitted using least squares linear regression and b represents the slope of the line. (b) The displacement curve of oestradiol-17 β concentrations of serially diluted elephant plasma (∘ b = -0.83) was parallel ($t_5 = 2.25, p > 0.05$) to that of the oestradiol-17 β standards (• b = -0.65).

Results

Progesterone

The concentrations of progesterone measured in the plasma from non-pregnant females (n = 28) ranged from the detection limit of the assay to 2 910 pg/ml (mean ± s.e.m., 730 ± 150 pg/ml, Fig. 4a). Within the group of non-pregnant females, there were no significant differences ($p > 0.05$) in the concentrations of progesterone in subadult (mean ± s.e.m., 560 ± 140 pg/ml, n = 9) and lactating (720 ± 180 pg/ml, n = 18) females. Concentrations in plasma from pregnant females (n = 65) ranged from 221 to 3 363 pg/ml (Fig.4a), with a mean ± s.e.m. of 1 110 ± 80 pg/ml. Mean progesterone concentrations for early- (1 090 ± 100 pg/ml, n = 28), mid- (1 360 ± 390 pg/ml, n = 8) and late-pregnant (620 ± 110 pg/ml, n = 29) animals were similar ($p > 0.05$). Plasma concentrations of progesterone as a function of gestational age varied considerably and did not follow a definable trend during pregnancy (Fig. 4a). However, the mean progesterone concentration in peripheral plasma from all the pregnant cows were significantly higher ($Z(U=360)= -3.24$, $p < 0.05$) than that for all the non-pregnant cows.

Concentrations of progesterone measured in foetal plasma ranged from 547 to 4 876 pg/ml (mean ± s.e.m, 1 630 ± 200 pg/ml, n = 26). Blood was collected from foetuses between the ages of 6.1 and 20.6 months, but there was only one individual in the early-foetus category. (This class was excluded from subsequent statistical analysis.) There were no significant differences ($p > 0.05$) between concentrations measured for mid- and late-foetuses and therefore no trends through the gestation period could be showed. The plasma progesterone levels of mid-pregnant females and their foetuses, and late-pregnant females and late-foetuses were similar ($p > 0.05$). Foetal plasma

progesterone concentration was significantly higher than that of non-pregnant ($Z(U=91)= -3.894$, $p < 0.05$) and of pregnant ($Z(U=543.5)= -2.65$, $p < 0.05$) females. There was no obvious relationship between foetal and maternal progesterone concentrations (Fig. 4b).

5α-dihydroprogesterone

Concentrations of 5α-dihydroprogesterone measured in the plasma of non-pregnant ($n = 21$) females ranged between 34 and 4 112 pg/ml, with a mean ± s.e.m. of 628 ± 127 pg/ml (Fig. 5a). Concentrations in subadult (693 ± 280 pg/ml, $n = 6$) and lactating (603 ± 150 pg/ml, $n = 15$) females were similar ($p > 0.05$). Plasma concentrations of 5α-dihydroprogesterone in pregnant females ranged from 224 to 12 053 pg/ml (Fig. 5a), with mean values for early- (3 765 ± 477 pg/ml, $n = 29$), mid- (3 175 ± 718 pg/ml, $n = 9$) and late- (2 653 ± 407 pg/ml, $n = 35$) pregnant females not differing statistically ($p > 0.05$). As for progesterone, plasma concentrations of 5α-dihydroprogesterone in pregnant females did not follow a definable temporal trend during pregnancy. Further analysis showed that the mean plasma concentration of 5α-dihydroprogesterone for all pregnant females (3 159 ± 289 pg/ml, $n = 73$) was significantly higher ($Z(U=145)= -4.972$, $p < 0.05$) than that for all the non-pregnant animals (628 ± 127 pg/ml, $n = 23$).

Concentrations of 5α-dihydroprogesterone in foetal plasma ($n = 16$) ranged from 337 to 2 149 pg/ml. The concentrations varied considerably between individual foetuses and no temporal trend could be detected. The mean 5α-dihydroprogesterone concentration in foetal plasma (± s.e.m., 1 013 ± 112 pg/ml) was significantly higher ($Z(U=46)= -2.389$, $p < 0.05$) than that for non-pregnant females, but significantly lower ($Z(U=126)= -4.021$, $p < 0.05$) than that for all the pregnant females.

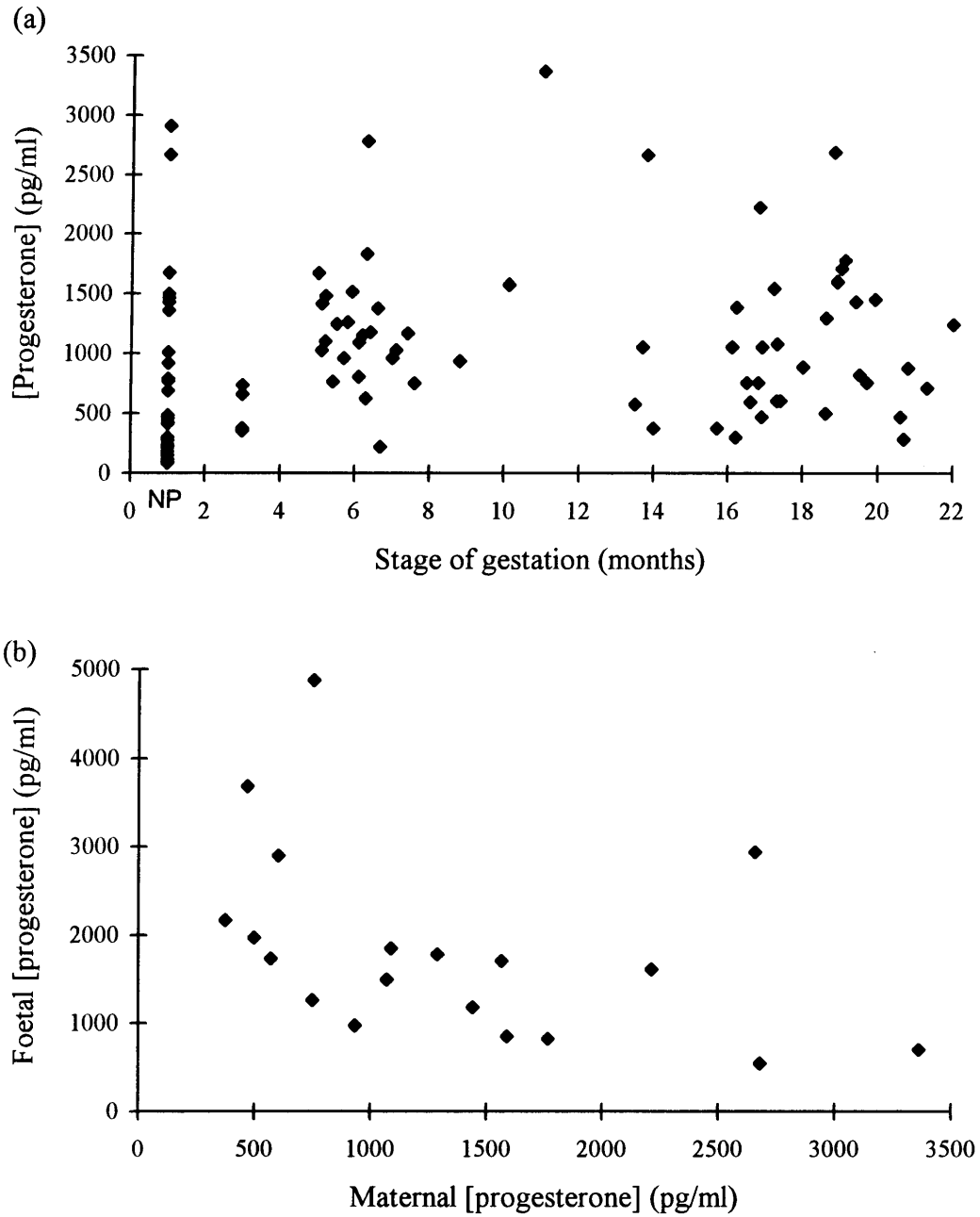

Figure 4 (a) Plasma concentrations of progesterone measured in non-pregnant and pregnant elephant cows. All the cows that carried embryos or foetuses too small to weigh are grouped together at 3 months. (NP = non-pregnant females). (b) A scatterplot to show the relationship between the concentrations of progesterone in maternal and foetal plasma.

However, no obvious relationship between foetal 5α-dihydroprogesterone and maternal 5α-dihydroprogesterone (Fig. 5b) was observed.

There were no significant differences ($p > 0.05$) and a significant positive linear relationship ($t_{16} = 3.824$, $p<0.05$) between the circulating concentrations of progesterone and 5α- dihydroprogesterone in non-pregnant females (Fig. 6a). For pregnant animals, the plasma concentrations of 5α-dihydroprogesterone were significantly higher ($Z(U=541)= -6.3$, $p < 0.05$) than those for progesterone. However, no relationship between the concentrations of the two progestins (Fig. 6b) could be determined. There were no significant differences ($p > 0.05$) or relationships between the concentrations of progesterone and 5α-dihydroprogesterone in foetal plasma.

Oestradiol-17β

Concentrations of oestradiol-17β in the plasma of non-pregnant females ($n = 9$) ranged from the detection limit of the assay to 35.7 pg/ml (Fig.7), with a mean ± s.e.m. of 15.3 ± 3.7 pg/ml. The concentrations of oestradiol-17β measured in plasma from pregnant females ($n = 14$) were between the limit of detection and 64.7 pg/ml, with a mean ± s.e.m. of 23.5 ± 4.6 pg/ml (Fig. 7). Mean concentrations for pregnant animals were not significantly different ($p>0.05$) from that for the non-pregnant females. Almost half (40% for non-pregnant females and 43% for pregnant females) of the measured concentrations were below the detection limit of the assay. Furthermore, the plasma concentrations of oestradiol-17β in pregnant females did not follow any temporal trends. The concentrations of oestradiol-17β in foetal plasma ($n = 6$) ranged between the lower limit of

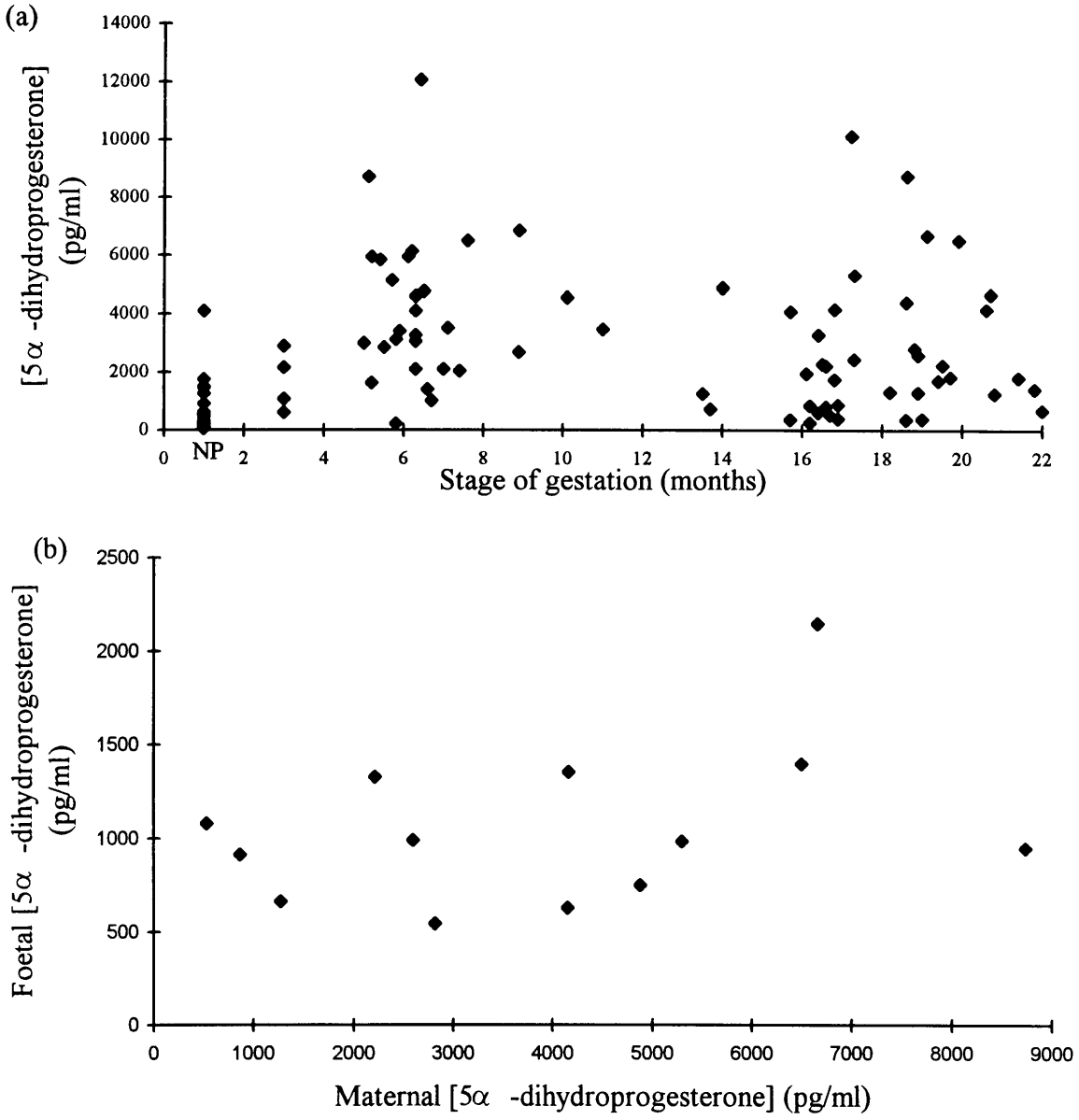

Figure 5 (a) Plasma concentrations of 5α -dihydroprogesterone measured in non-pregnant and pregnant elephant cows. All the cows that carried embryos or foetuses too small to weigh, are grouped together at 3 months (NP = non-pregnant females). (b) A scatterplot to show the relationship between the concentrations of 5α -dihydroprogesterone in maternal and foetal plasma.

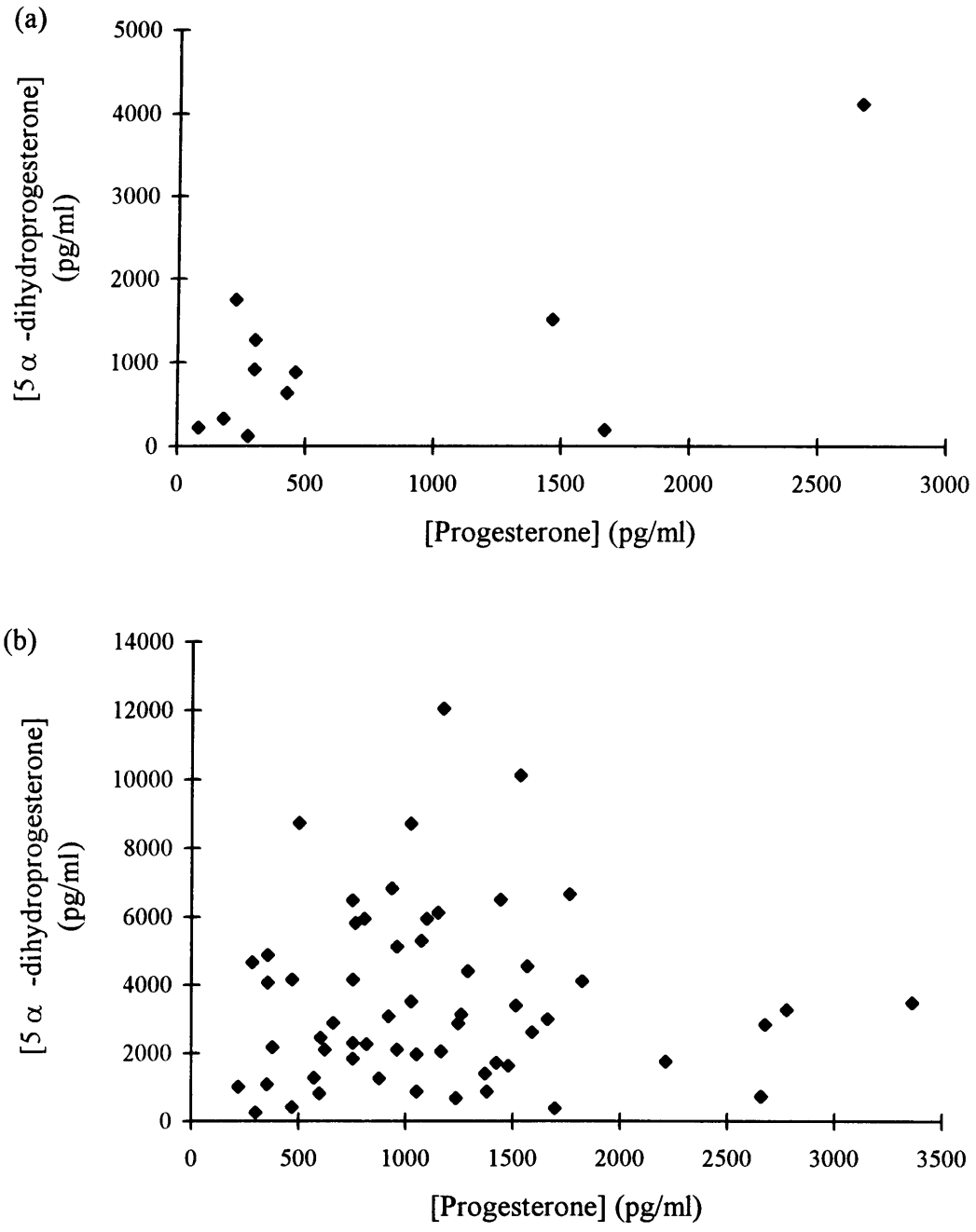

Figure 6 Scatterplots to show the relationship between the circulating concentrations of 5α-dihydroprogesterone and progesterone for (a) non-pregnant and (b) pregnant elephant cows.

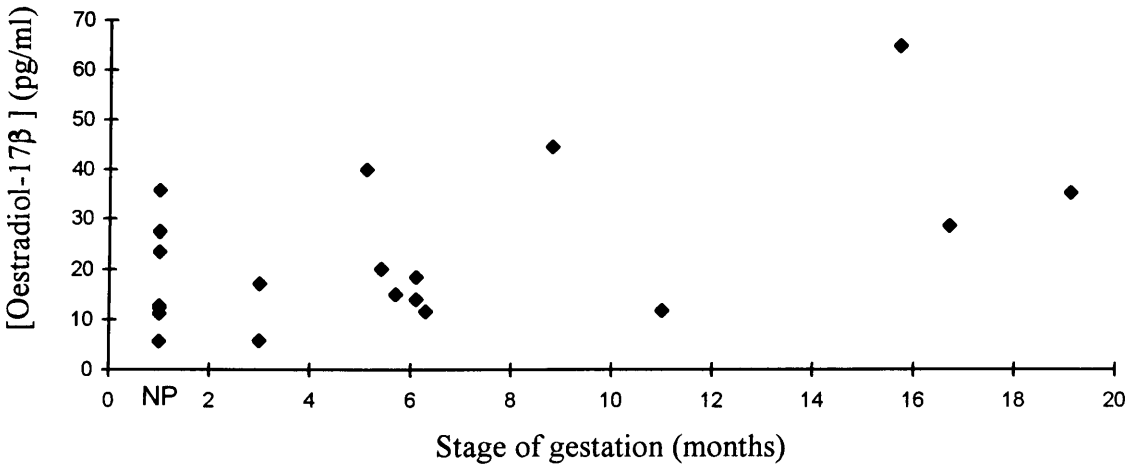

Figure 7 Plasma concentrations of oestradiol-17β measured in non-pregnant and pregnant elephant cows. Cows with embryos or foetuses too small to weigh are grouped together at 3 months (NP = non-pregnant females).

detection of the assay and 58.5 pg/ml, with a mean ± s.e.m. of 29.6 ± 8.9 pg/ml, which did not differ significantly (p>0.05) from that of non-pregnant or pregnant females. As with maternal plasma oestradiol-17β, the concentrations of the steroid in foetal plasma varied greatly between individuals and no temporal trend could be detected.

Discussion

Mean plasma concentrations of progesterone measured in the present study were similar to the values reported previously (Plotka *et al.* 1975; McNeilly *et al.* 1983; Brannian *et al.* 1988; Plotka *et al.* 1988; De Villiers *et al.* 1989; Kapustin *et al.* 1996; Schwarzenberger, Strauβ, Hoppen, Schaftenaar, Dieleman, Zenker & Pagan 1996; Hodges *et al.* 1997, see Table 1). Slight between-study differences may be ascribed to differences in the cross-reactivities of the antibodies used by the different groups of researchers. In spite of these differences it is important to note that the values reported in all these studies were considerably lower than some of those recorded in many other mammals (Heap 1972). For instance plasma progesterone concentrations can reach 35 ng/ml in the ferret, 90 ng/ml in the chimpanzee, 300 ng/ml in the guinea pig (Heap 1972), 33 ng/ml in the horse (Holtan, Houghton, Silver, Fowden, Ousey & Rossdale 1991) and 200 ng/ml in the human (Heap 1972; Johnson & Everitt 1988) during pregnancy. The low concentrations measured in elephant plasma therefore supports the early notions that a hormone other than progesterone may support pregnancy (Short & Buss 1965; Hodges *et al.* 1994; Schwarzenberger *et al.* 1996; Trohorsch *et al.* 1996), or that the elephant is extremely sensitive to progesterone (Hanks & Short 1972; Plotka *et al.* 1975).

Table 1 Plasma progesterone concentrations recorded in African elephants.

Reproductive status	Mean ± s.e.m. plasma progesterone (pg/ml)	Range of progesterone concentration (pg/ml)	Sources
Non-pregnant		210 - 220	Plotka *et al.* 1975
Non-pregnant:			
< 13 years old	320 ± 50	120 - 600	McNeilly *et al.* 1983
> 13 years old	710 ± 80	90 - 1 500	"
Non-pregnant:			
luteal phase		20 - 280	Brannian *et al.* 1988
Non-pregnant	330 ± 10	<50 - 680	Plotka *et al.* 1988
Non-pregnant	720 ± 270	210 - 1 280	De Villiers *et al.* 1989
Non-pregnant:			
luteal phase	540 ± 40	500 - 1 000	Schwarzenberger *et al.* 1996
follicular phase		<100	"
Non-pregnant:			
luteal phase		400 - 800	Kapustin *et al.* 1996
non-luteal phase		<80	"
Non-pregnant	730 ± 150	10 - 2 910	Present study
Pregnant & non-pregnant		260 - 1 130	Hodges *et al.* 1997
Pregnant		420 - 480	Plotka *et al.* 1975
Pregnant	1 410 ± 90		McNeilly *et al.* 1983
Pregnant	660 ± 340	80 - 1 300	De Villiers *et al.* 1989
Pregnant	1 110 ± 80	220 - 3 360	Present study

In agreement with McNeilly *et al.* (1983), the present study recorded significantly lower mean plasma progesterone concentrations in non-pregnant females than in pregnant females. This is in contrast to the reports of De Villiers *et al.* (1989) and Hodges *et al.* (1997). However, most of the females in both the pregnant and non-pregnant groups (80% and 87% respectively) had plasma progesterone concentrations less than 1 500 pg/ml. The determination of reproductive status (i.e pregnant versus non-pregnant) can therefore not be based on plasma progesterone concentrations alone. A similar overlap in ranges of concentrations for pregnant and non-pregnant animals was reported by De Villiers *et al.* (1989) and McNeilly *et al.* (1983). The higher concentrations measured in foetal plasma (mean ± s.e.m., 1 630 ± 200 pg/ml), compared to plasma from pregnant females (mean ± s.e.m., 1 110 ± 80 pg/ml), suggests that there may be foetal production of the hormone, although the opposite has been suggested by Hodges *et al.* (1997).

The plasma concentrations of 5α-dihydroprogesterone measured in the present study were lower than those reported by Hodges *et al.* (1997). Although absolute values differ, the trends reported by Hodges *et al.* (1997) seem to be comparable to those of the present study, with levels of 5α-dihydroprogesterone being higher in pregnant females than in non-pregnant animals. However, the decline in plasma concentrations during late pregnancy suggested by Hodges *et al.* (1997) could not be confirmed in the present study. Some of the plasma samples (n = 12) were included in another analysis (Greyling *et al.* 1998), where an increase in circulating concentrations of 5α-dihydroprogesterone with gestation was illustrated. However, if all the plasma samples were included in the analysis (as in the present chapter), similar trends could not be shown. This lack of repeatability in trends may be ascribed to large individual variation in plasma concentrations.

Even though the concentrations of 5α-dihydroprogesterone in the pregnant animals were significantly higher than the concentrations of progesterone in the same samples, the circulating levels for both these progestins did not follow definable trends throughout pregnancy, and both were characterised by marked variation at a specific stage of pregnancy. There was also no obvious relationship between the plasma concentrations of progesterone and 5α-dihydroprogesterone in pregnant females. Circulating levels of the two progestins were quite similar in the non-pregnant animals, but 5α-dihydroprogesterone concentrations increased with the onset of pregnancy and were maintained at this elevated level throughout gestation. However, this pregnancy-associated rise did not follow predictable trends, and the values often overlapped with the concentrations measured in non-pregnant cows.

5α-Dihydroprogesterone has some progestogenic activity, but it can not maintain pregnancy in rats (Chatterton 1982). Progestational activity of a molecule are determined by a few factors, including a high electron density between C_4 and C_5 and an acetyl radical that must be present at $C_{17\beta}$. The α-face of a steroid molecule is involved in the association with proteins (i.e. receptors, Dorrington 1977). This association of 5α-dihydroprogesterone with receptors, which supports biological significance, was illlustrated for the African elephant by the high (43%) relative binding affinity between 5α-dihydroprogesterone and the endometrial progesterone receptors (Greyling *et al.* 1997). The biological significance of this progestin is also suggested by the ability of 5α-dihydroprogesterone to down-regulate these receptors (Greyling *et al.* 1998).

The concentrations of oestradiol-17β measured were consistently low, regardless of the reproductive status of the females. There were also no patterns associated with foetal age. The concentrations measured in the present study are similar to those reported by previous workers (Plotka *et al.* 1975; Hodges *et al.* 1987; Plotka *et al.* 1988; Kapustin *et al.* 1996, see Table 2). Much higher circulating concentrations of oestradiol-17β have however been reported by Hodges *et al.* (1983) and Brannian *et al.* (1988).

Conclusion

Although there were significant differences between the concentrations of progesterone and 5α-dihydroprogesterone in pregnant and non-pregnant cows, these concentrations were characterised by individual variation. Measurements of neither progesterone nor 5α-dihydroprogesterone can therefore be used to predict the reproductive status in elephant cows and more applicable methods should be investigated. It has been suggested that plasma concentrations of prolactin can be used to diagnose pregnancy in African elephants (McNeilly *et al.* 1983; Hodges, McNeilly & Hess 1987). The measurement of urinary levels of 5α-reduced progestins, particularly 5α-pregnane-3α-ol-20-one, in African elephants (Trohorsch *et al.* 1996; Heistermann, Trohorsch & Hodges 1997) and 5α-pregnanetriol in Asian elephants (Niemuller *et al.* 1993) and feacal levels of progestins (Wasser *et al.* 1996) and 5α-pregnane-3α-ol-20-one (Heistermann, Fieβ & Hodges 1997) may provide reliable methods for monitoring ovarian function. However, these suggestions are based on limited numbers of females, which are often housed in zoological gardens without contact with male elephants and does not reflect intra-individual differences. The applicability of these methods to free-ranging animals is uncertain. Studies based on serial samples may show trends in the concentrations of

Table 2 Plasma oestradiol-17β concentrations recorded in African elephants

Reproductive status	Mean (±s.e.m.) plasma oestradiol-17β (pg/ml)	Range of oestradiol-17β concentration (pg/ml)	Sources
Non-pregnant		9- 6	Plokta *et al.* 1975
Non-pregnant	157 ± 6	120-186	Hodges *et al.* 1983
Non-pregnant	6.9 ± 0.7	<0.8-22	Hodges *et al.* 1987
Non-pregnant		<4-52	Brannian *et al.* 1988
Non-pregnant		0-13.6	Plotka *et al.* 1988
Non-pregnant		0-24	Kapustin *et al.* 1996
Non-pregnant	15.32 ± 3.74	3.9-35.72	Present study
Pregnant		11-20	Plokta *et al.* 1975
Pregnant	498.9 ± 72.3	116-1428	Hodges *et al.* 1983
Pregnant:			Hodges *et al.* 1987
0-6 months	14.4 ± 3.7	3.5-38	
7-22 months	86.0 ± 6.5	35-212	
Pregnant	23.49 ± 4.6	3.9-64.66	Present study

progestins during pregnancy and in comparison with non-pregnant females, while those based on individual samples fail to illustrate these definite trends, due to differences between individuals.

The low circulating concentrations of oestradiol-17β suggest that, as with progesterone, it may not be the most biologically active oestrogen and that it may be more important and significant to measure another oestrogen, conjugated steroid or derivative thereof as is suggested in Hodges *et al.* (1983).

CHAPTER 3

Steroidogenic potential of the placenta and corpora lutea

Introduction

Reproductive steroids are produced from cholesterol, which undergoes side-chain cleavage to produce pregnenolone, a reaction catalised by a cytochrome P450 (Erickson 1987; Miller 1988). The synthesis of progesterone from pregnenolone is a two-step reaction catalysed first by Δ^5 - 3β hydroxysteroid dehydrogenase, for the dehydroxylation of the 3β-hydroxy-5-ene-steroid, and secondly by $\Delta^{5\rightarrow4}$ isomerase for the isomerisation of the 3-oxo-5-ene-steroid. In the next step of this steroid biosynthetic pathway, progesterone is reduced to 17α-hydroxyprogesterone. The oestrogens and androgens are also produced by further steps in this pathway (Cheesman 1982; Ishii-Ohba, Inano & Tamaoki 1986; Miller 1988). These enzymes have multisubstrate specificity (Takahashi, Luu-The & Labrie 1990) and are inhibited competitively by a variety of natural and synthetic substances (Heap, Gombe & Sale 1975; Jung-Testas, Hu, Baulieu & Robel 1989; Baggia, Albrecht & Pepe 1990; Takahashi *et al.* 1990; Wanatabe, Hirato, Hashino, Kosaki, Kimura, Nakayama & Yanaihara 1990; Vega, Devoto, Castro & Kohen 1994).

3β-Hydroxysteroid dehydrogenase occurs on smooth endoplasmic reticulum (Girmus & Ledwitzrigby 1987) and its activity is widespread among tissues, including human placenta, developing and antral follicles (Sasano, Mori, Sasano, Nagura & Mason 1990), as well as in mouse

stomach, liver, kidney, lung, spleen and seminal vesicles (Colombo, Belvedere & Simontacchi 1989).

Sites of progestin metabolism, other than the ovary, include the adrenal cortex (eg. guinea pig, Demura, Driscoll & Strott 1990), mouse thymus (Weinstein, Linder & Eckstein 1977), morulae and pre-implantation blastocysts (Wu & Liu 1990). Progestins have also been called neurosteroids, due to their presence and production in the neuroendocrine system. Rat glial cells, prepared from forebrains, convert pregnenolone to progesterone, 5α-dihydroprogesterone and 3α-hydroxy-5α-pregnan-20-one (Jung-Testas *et al.* 1989), while 5α-dihydroprogesterone and 3α,5α-tetra-hydroprogesterone are the main products from progesterone metabolism in the anterior pituitary and hypothalamus (Karavolas & Hodges 1990).

The ability of the placenta to synthesise and metabolise steroid hormones varies between species (Strauss *et al.* 1996). In humans (Cheesman 1982; Khan-Dawood 1987), rhesus monkeys, guinea pigs and horses the placenta produces sufficient progesterone to maintain pregnancy (Heap 1972). The placenta of the rat (Townsend & Ryan 1970) and olive baboon (Wango & Mbui 1994) produce progesterone from pregnenolone. In the rat (Sanyal & Villee 1973), horse (Hamon *et al.* 1991), human (Ehlers, Holbrook, Griffing, Abaoge, Edelink & Melby 1987) and olive baboon (Wango & Mbui 1994; Waddell, Pepe & Albrecht 1996) placental progesterone is further metabolised, in the rat to 3β-hydroxy-5α-pregnan-20-one (Sanyal & Villee 1973), in the human to 19-hydroxyprogesterone (Ehlers *et al.* 1987) and in the horse to 5α-dihydroprogesterone (Hamon *et al.* 1991). No published information on the metabolic activities of the placenta of the African elephant

is yet available. The ability of the elephant placenta and corpora lutea to metabolise pregnenolone, and thereby to contribute to the levels of circulating steroids is examined in the present chapter.

Materials and methods

Determination of the retention times of selected steroids on the high pressure liquid chromatography (HPLC) column

To determine the elution positions of pregnenolone and progesterone, 10µl each (approximately 4 000 dpm) of ^{3}H-pregnenolone and ^{3}H-progesterone were injected into a reversed phase C18 HPLC column (150mm x 4.6mm Lichrosorb, Phenomenex, Torrence, U.S.A.) to which a precolumn (µBondapak C18, Waters, Millipore, Milford, U.S.A.) was attached. The HPLC system (Waters, Milford, U.S.A) consisted of a 590 programmable solvent delivery module, an automated gradient controller and a 712 WISP autoinjector. The steroids were eluted with acetonitrile:water (55:45 v/v) at a flow rate of 1ml/min. The acetonitrile (Romil Chemicals, Cambridge, U.K.) and water (double distilled in glass and deionised) were filtered through a 0.45 micron filter (Millipore, Milford, U.S.A.) prior to use. Sixty fractions of 0.5ml each were collected into scintillation vials, 4ml of scintillation cocktail were added to each vial and the radioactivity of each fraction determined by scintillation counting.

Approximately 1mM each of unlabelled progesterone, 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 5α-dihydroprogesterone, 5β-dihydroprogesterone and 5α-pregnan-3α-ol-20-one were injected separately into the HPLC system. An ultraviolet detector (Programmable multiwavelength detector,

M490, Waters, Milford, U.S.A.) was attached to the HPLC system to determine the retention times of these unlabelled hormones.

Determination of R_f values of selected steroids by thin layer chromatography (TLC)

Approximately 1mM each of 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone and progesterone were loaded onto thin layer chromatography plates (silica gel 60, F_{254}, 10 x 20 cm, Merck, Darmstadt, Germany). Two mobile phases, namely chloroform:ethyl acetate (9:1, chloroform from Sky Chemicals, Alberton, S.A., ethyl acetate from Robert Lundie, Westgate, S.A.) and n-hexan:ethyl acetate (5:2, n-hexan from Merck, Darmstadt, Germany) were used to determine their R_f values. The position of progesterone on the plates were visualised under ultraviolet light (Spectroline TC-312A transilluminator, 312nm ultraviolet, New York, U.S.A.). The plates were then sprayed with 70% H_2SO_4 (Glassworld, Newcastle, S.A) and placed at 110°C for 5 min. to char the other progestins. R_f values were subsequently determined from the positions of the steroid standards.

Collection of study material

Material was collected from elephants (n = 13) culled in the Kruger National Park during the population control programme of 1995. Whole ovaries and pieces of placenta were removed within 30 min. of death, put into labeled plastic bags, and placed on ice for transport to the laboratory where the incubation experiments were conducted. All incubations commenced within two hours of the collection of study material.

Incubations

Incubation vials were prepared prior to the collection of study material each day. Each vial contained 2 ml Eagle's minimum essential medium (with Earle's salts, L-glutamine, 25 mM HEPES and 0.2% sodium bicarbonate, pH 7.4, Highveld Biological, Kelvin, S. A.) and 100µl ^{3}H-pregnenolone ([7-^{3}H(N)]-pregnenolone, specific activity 21.1 Ci/mmol, New England Nuclear, Boston, U.S.A., ca. 140 000dpm). Placental tissue was cut into pieces weighing approximately 0.5g each, washed with 0.9% saline to remove excess blood, and placed into the incubation vials. The vials were placed in a shaking water bath at 37°C for three hours. Corpora lutea were removed from the ovaries and weighed. Slices weighing approximately 0.5g each were placed into the incubation vials and incubated at 37°C in a shaking water bath for two hours. The incubations were terminated by the addition of 8 ml absolute ethanol. The vials were capped and stored at -20°C. Control vials containing Eagle's minimum essential medium (2 ml) and ^{3}H-pregnenolone (100µl) were incubated alongside the placenta and corpus luteum incubations, also at 37°C for two hours.

Tissue extraction

Tissues were chopped finely with a clean scalpel blade and then homogenised with a Ultra-Turrax homogeniser (Janke & Kunkel KG, Staufen, Germany) in the medium-ethanol mixture. The homogenising probe was washed with 2 ml absolute ethanol, which was added to the vials. The vials were vortexed, centrifuged (4°C, 2 500 rpm, 15 min.), the supernatants decanted and evaporated under N_2 at 37°C. The pellets were re-extracted with 5 ml diethyl ether, vortexed, centrifuged (4°C, 2 000 rpm, 10 min.), placed at -20°C for 60 min. and then at -70°C for 10 min. The supernatants were decanted into the tubes which contained the dried ethanol extracts and

evaporated. The dried extracts were reconstituted in 1 ml absolute ethanol. An aliquot (200µl) was placed in a scintillation vial with 4 ml scintillation cocktail and the radioactivity counted to determine the extraction efficiency. The mean extraction efficiency for placental tissue was 76.1 ± 2.1% (n = 21) and that for luteal tissue 66.3 ± 1.0% (n = 20).

High pressure liquid chromatography (HPLC) of tissue samples

The extracts (100µl each) of the placenta and corpus luteum incubations were applied to the HPLC system described previously. Sixty fractions of 0.5 ml each were collected into scintillation vials, 4ml of scintillation cocktail were added to each vial and the radioactivity of each fraction determined by scintillation counting. The mean (± s.e.m.) recovery of radioactive compounds from the HPLC column was 90.3 ± 1.7% (n = 42).

Determination of HPLC peaks

A HPLC fraction was considered a peak if the radioactivity (in dpm) was equal to or exceeded four standard deviations of the mean background radioactivity per fraction. Furthermore, the radioactivity in the fraction had to exceed that of the fraction before and after it.

Thin layer chromatography (TLC) of selected metabolites

The fractions that contained some of the metabolites (11α-hydroxyprogesterone, 17α-hydroxyprogesterone and 20α-dihydroprogesterone) were pooled and extracted with 4 ml diethyl ether, as described for the extraction of steroids from plasma (Chapter 2). The pellets were re-extracted with 2 ml diethyl ether and the supernatants of the two extraction steps were pooled.

These were evaporated under N_2 at 37°C and reconstituted in 100µl absolute ethanol. Two aliquots (10µl each) were loaded separately onto thin layer chromatography plates. The same two mobile phases that were used for the steroid standards, namely chlorofom:ethyl acetate (9:1) and n-hexan:ethyl acetate (5:2), were used to separate these metabolites. The plates were visualised under ultraviolet light and also sprayed with 70% H_2SO_4 and placed at 110°C for 5 min. to determine the positions of the metabolites. R_f values could then be calculated.

Results

Retention times for hormone standards

The elution profiles for the tritiated standards (^{3}H-pregnenolone and ^{3}H-progesterone) show that pregnenolone eluted in fractions 33-37 (Fig. 8a) and that progesterone eluted in fractions 29-32 (Fig 8b). These elution positions correspond to retention times of 18 min. for ^{3}H-pregnenolone and 15.5 min. for ^{3}H-progesterone. The retention times for the unlabelled hormones (11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, progesterone, 5α-dihydroprogesterone, 5β-dihydroprogesterone and 5α-pregnan-3α-ol-20-one) are presented in Table 3.

Control incubations

The elution profile (Fig. 8c) of the control incubations show a single peak that eluted consistently in fractions 33-37. This represents the unmetabolised pregnenolone that was added to the incubation vials.

Table 3 Retention times of unlabelled 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, progesterone, 5β-dihydroprogesterone, 5α-pregnan-3α-ol-20-one and 5α-dihydroprogesterone from the HPLC column.

Hormone	Retention time (min.)
11α-Hydroxyprogesterone	4.2
17α-Hydroxyprogesterone	6.5
20α-Dihydroprogesterone	10.3
20β-Dihydroprogesterone	13.9
Progesterone	15.5
5β-Dihydroprogesterone	20.9
5α-Pregnan-3α-ol-20-one	23.2
5α-dihydroprogesterone	25.0

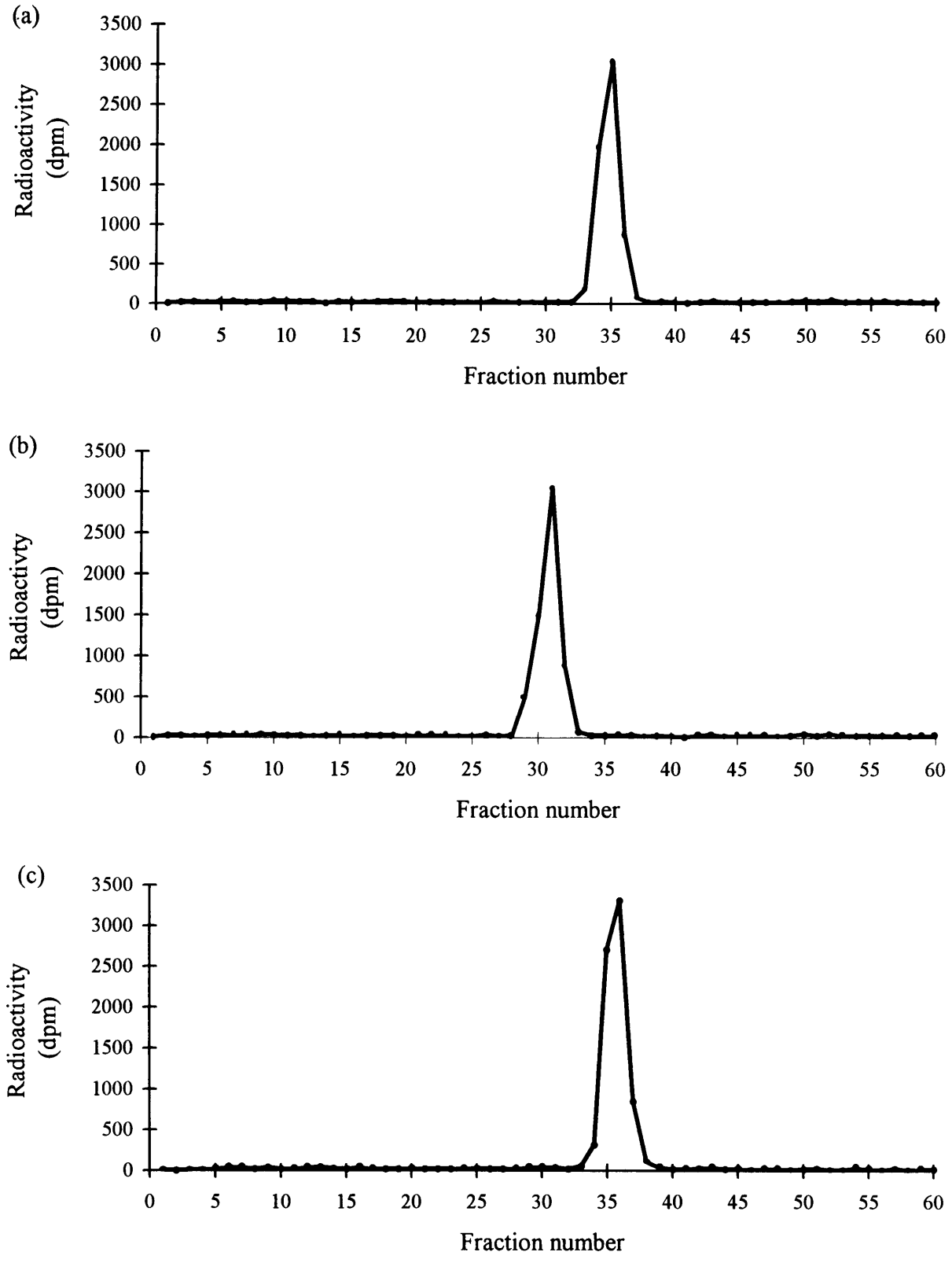

Figure 8 Elution profiles of the (a) ^{3}H-pregnenolone standard, (b) ^{3}H-progesterone standard and (c) ^{3}H-pregnenolone that was incubated in medium, in the absence of tissue.

R_f values for hormone standards

The R_f values of 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone and 20β-dihydroprogesterone that were determined with two mobile phases on thin layer chromatography, are presented in Table 4.

Placenta incubations

The amount of ^{3}H-pregnenolone converted by placenta ranged from 17.0 - 65.0%, with the highest conversion occurring during mid-pregnancy. Mean ± s.e.m percentage conversions for each reproductive class (early-, mid- and late-pregnant) are presented in Table 5. Radiolabelled pregnenolone was converted to two main and three minor products (Table 5). The principal products eluted in fractions eight to ten (11α-hydroxyprogesterone) and fractions 13-16 (17α-hydroxyprogesterone) respectively and the minor products in fractions 24-27 (20β-dihydroxyprogesterone), fractions 42-45 (5α-pregnane-3α-ol-20-one) and fractions 49-52 (5α-dihydroprogesterone) respectively (Fig. 9).

The percentage conversion of pregnenolone to 11α-hydroxyprogesterone ranged between 0 and 18.0%, with the mean for mid-pregnant females the highest (14.9 ± 5.4%). The percentage of pregnenolone converted to 17α-hydroxyprogesterone ranged from 3.0 to 33.0% and the placentae from mid-pregnant females had the highest mean conversion (14.4 ± 9.4%). 20β-Dihydroprogesterone was not produced in any incubation with placentae from early-pregnant females and only small amounts of it were produced by one female each in the mid- (3.6% conversion) and late-pregnant (1.5% conversion) category. 5α-Pregnane-3α-ol-20-one was only

Table 4 R_f values of 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone and 20β-dihydroprogesterone that were determined with two mobile phases on TLC.

Hormone	R_f values	
	Chloroform:ethyl acetate	n-hyxan:ethyl acetate
11α-hydroxy - progesterone	0.04	0.03
17α-hydroxy - progesterone	0.17	0.15
20α-dihydro - progesterone	0.21	0.17
20β-dihydro - progesterone	0.54	0.2

Table 5 Percentage conversion of ^{3}H-pregnenolone and production of metabolites by placental tissue incubated for three hours at 37°C. The number of animals used in each reproductive class is indicated in parenthesis and each value is followed by one s.e.m.

Reproductive status	^{3}H-pregnenolone converted (%)	Metabolites produced (%)				
		11α-hydroxy-progesterone	17α-hydroxy-progesterone	20β-dihydro-progesterone	5α-pregnane-3α-ol-20-one	5α-dihydro-progesterone
Early-pregnant (n = 6)	26.2 ± 1.6	11.4 ± 2.5	8.3 ± 1.2	0	0	2.9*
Mid-pregnant (n = 3)	38.0 ± 13.5	14.9 ± 5.4	14.4 ± 0.9	3.6*	0	3*
Late-pregnant (n = 4)	21.2 ± 3.3	6.5 ± 2.4	5.3 ± 0.9	1.5*	5.3*	0

* Compound only recorded for one animal.

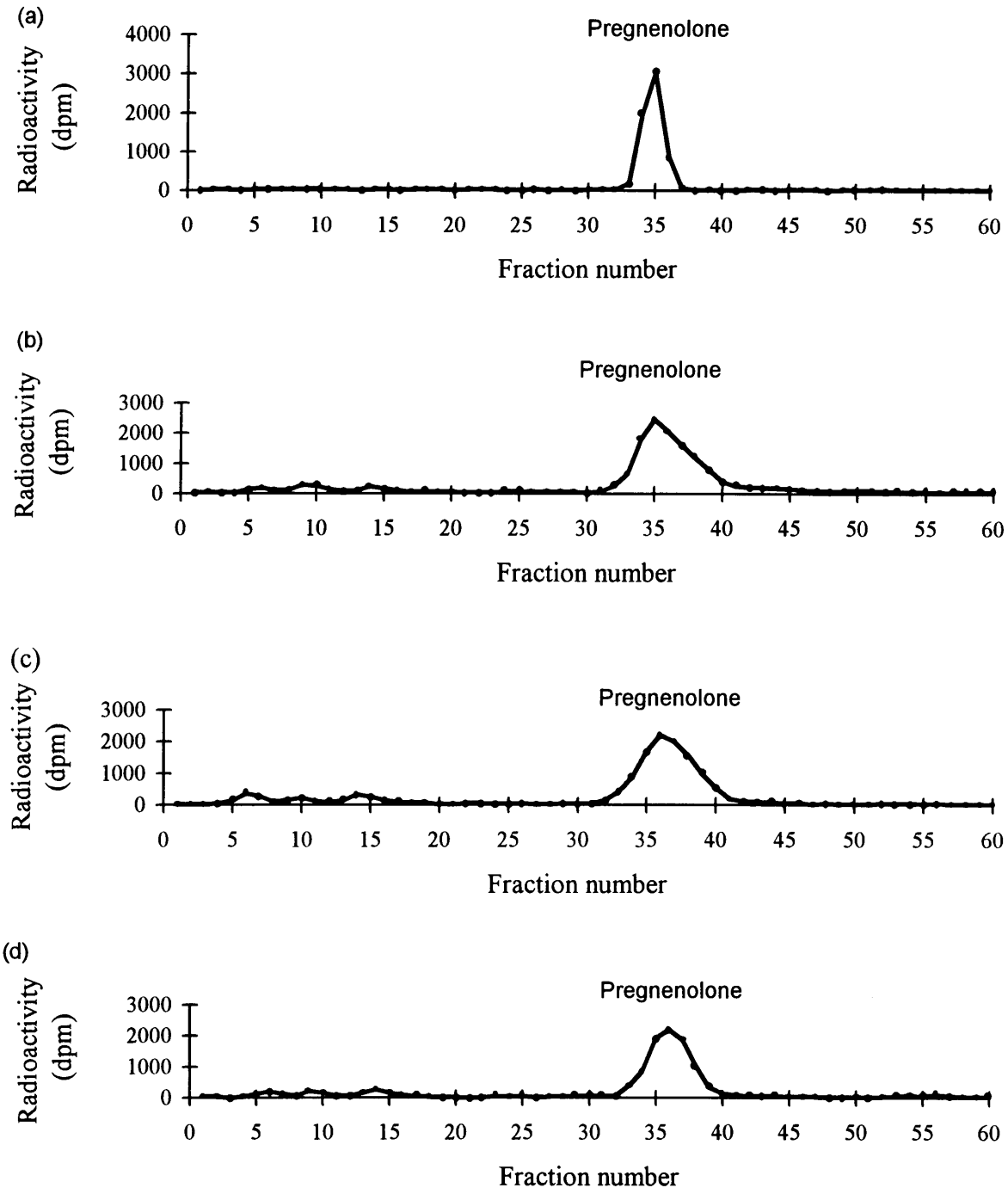

Figure 9 Elution profiles of (a) the control and placenta incubations from (b) an early-, (c) a mid- and (d) a late-pregnant elephant. The elution position of unmetabolised pregnenolone is indicated. The placentae were incubated for three hours.

produced by one animal in late-pregnancy (5.3% conversion). Two females, one in early- (2.9% conversion) and one in mid-pregnancy (3.0% conversion), produced 5α-dihydroprogesterone. Thus 20β-dihydroprogesterone, 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone are probably not typical conversion products of the placenta. The lowest percentage conversion to 11α-hydroxyprogesterone and 17α-hydroxyprogesterone were measured during late-pregnancy, which suggests a decline in the steroidogenic activity of the placenta towards the end of the gestation period.

Corpus luteum incubations

The conversion of ^{3}H-pregnenolone incubated with luteal tissue ranged from 0 to 86.9% (Table 6). Tritiated pregnenolone incubated with luteal tissue were converted to six metabolites. The most prominent conversion product of ^{3}H-pregnenolone eluted in the position of 5α-dihydroprogesterone (Fig. 10). Progesterone was produced by luteal tissue from three early-pregnant females (17.3, 16.5 and 8.3% conversion, respectively). 11α-Hydroxyprogesterone was produced by all the corpora lutea that were incubated (n = 20), with the percentage conversion ranging from 2.9% to 29.0%. The tritiated pregnenolone was also converted to 17α-hydroxyprogesterone, 5α-pregnane-3α-ol-20-one and 20α-dihydroprogesterone (fractions 19-23).

The conversion of 5α-dihydroprogesterone increased with gestation age, with means ± s.e.m. of 40.7 ± 6.5 %, 53.6 ± 11.4% and 77.4% (n = 1) produced during early-, mid- and late-pregnancy respectively. The highest conversion (8.8 ± 2.5%) of ^{3}H-pregnenolone to 11α-hydroxyprogesterone occurred during early-pregnancy and the lowest (7.5%) during late-pregnancy, although the mean

Table 6 Percentage (mean ± s.e.m.) conversion of ^{3}H-pregnenolone and production of metabolites by elephant corpora lutea incubated for two hours at 37°C. The number of animals used in each reproductive class is indicated in parenthesis.

		Metabolites produced (%)					
Reproductive stage	Pregenenolone converted (%)	Progesterone	11α-hydroxy-progesterone	17α-hydroxy-progesterone	5α-pregnane-3α-ol-20-one	5α-dihydro-progesterone	20α-dihydro-progesterone
Early-pregnant (*n*=13)	64.1±7.5	3.2 ± 1.8	8.8 ± 2.5	3.9 ± 1.1	5.1 ± 1.3	40.7 ± 6.5	0.4 ± 0.2
Mid-pregnant (*n*=6)	78.5±2.8	0	8.4 ± 2.8	1.6 ± 0.7	14.5 ± 10.5	53.6 ± 11.4	1.9 ± 1.2
Late-pregnant (*n*=1)	82.7	0	7.5	7.1	21.5	77.4	0

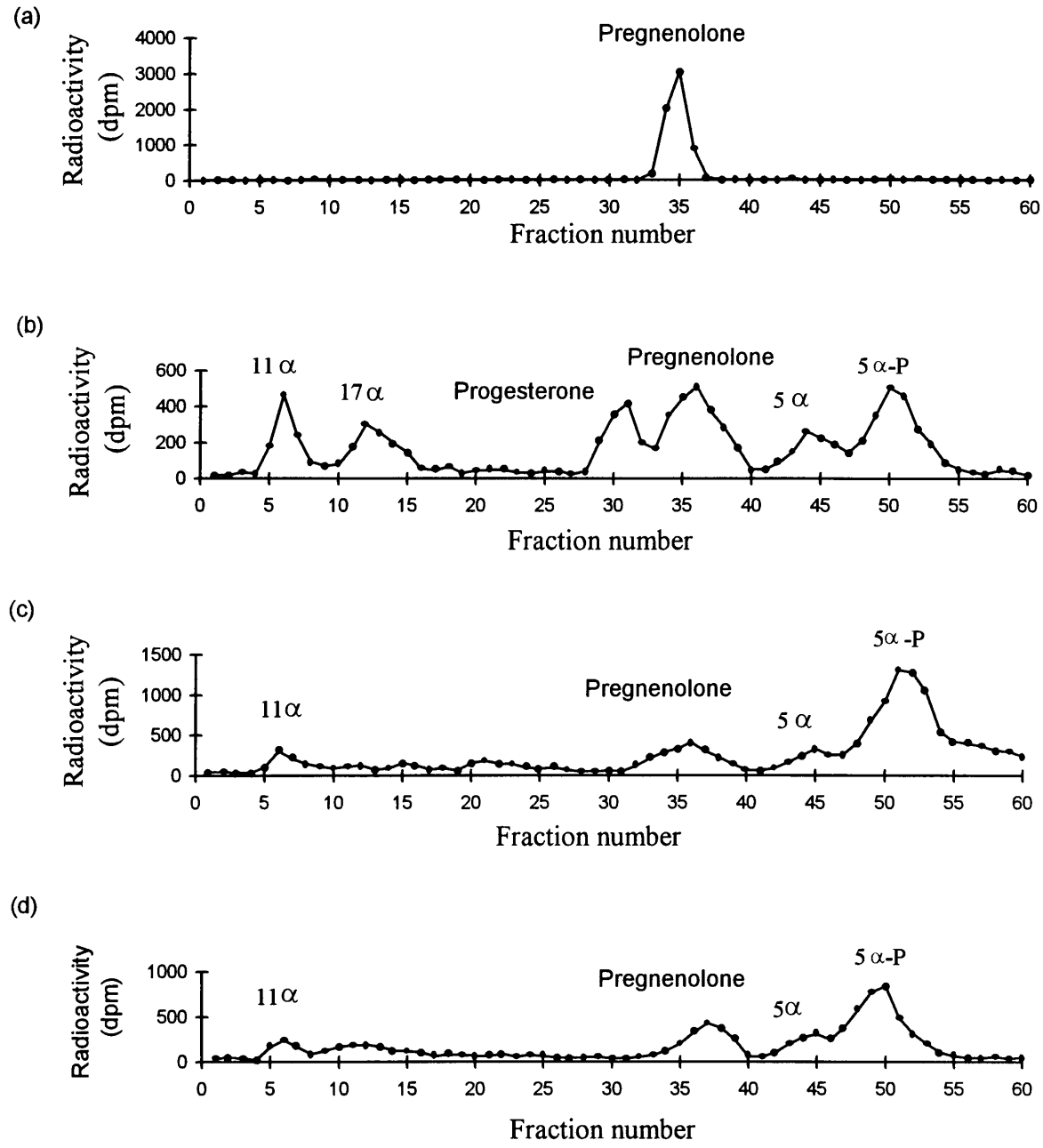

Figure 10 Elution profiles of (a) the control and corpus luteum incubations from (b) an early-, (c) a mid- and (d) a late-pregnant elephant. The elution positions of unmetabolised pregnenolone and the products [progesterone, 11 α -hydroxyprogesterone (11 α), 17 α-hydroxyprogesterone (17 α), 5 α-dihydroprogesterone (5 α) and 5 α -pregnan-3α -ol-20-one (5 α -P)] are indicated. The corpora lutea were incubated for two hours.

values for all the reproductive classes were similar (Table 6). The conversion of radiolabelled pregnenolone to 5α-pregnane-3α-ol-20-one varied from 4.4% to 21.5%, with the highest conversion occurring during late-pregnancy. Conversion to 17α-hydroxyprogesterone ranged between 2.1% and 23.0%, with the highest conversion measured in the corpus luteum from the late-pregnant female.

For some females (n = 6) more than one corpus luteum were used in the incubation experiments. The percentage conversion of tritiated pregnenolone and the metabolites produced by those corpora lutea from the same female were similar. There were two exceptions, namely the second corpus luteum from the left ovary of female E70 (0% ^{3}H-pregnenolone converted) and the third corpus luteum from the left ovary of female E85 (14.9% ^{3}H-pregnenolone converted). In both these cases the particluar luteal bodies were the smallest (Table 7) of those included in the incubation. They most probably were regressing corpora lutea of previous cycles or pregnancies and have lost most of their ability to metabolise pregnenolone.

Discussion

The ability of the elephant placenta to metabolise pregnenolone peaked during mid-gestation, when the highest (38.0 ± 13.5%) conversion of pregnenolone occurred. The most prominent metabolites produced were 11α-hydroxyprogesterone and 17α-hydroxyprogesterone with 20β-dihydroprogesterone, 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone quantitatively of lesser importance.

Table 7 Percentage conversion of ^{3}H-pregnenolone by different corpora lutea from the same female elephant. The tritiated pregnenolone were incubated with luteal tissue at 37°C for two hours.

Elephant number	Stage of pregnancy (months)	Corpus luteum code *	Corpus luteum weight (g)	^{3}H-pregnenolone converted (%)
E 44	6.3	R 1	19.1	68.0
		R 2	4.7	57.7
E 85	6.3	R 1	13.9	85.3
		R 2	10.6	77.3
		L 1	20.4	85.7
		L 2	10.2	86.9
		L 3	7.4	14.9
E 57	6.7	L 1	20.5	65.1
		L 2	12.1	80.4
E 70	7.6	L 1	12.7	80.0
		L 2	10.9	0
E 68	7.6	R 1	17.5	75.2
		R 2	11.2	73.4
		L 2	24.9	69.1
E 74	13.5	R 1	16.9	84.8
		R2	6.1	85.4
		L 1	18.6	83.1

*L = left ovary; R = right ovary

Two of these metabolites (11α-hydroxyprogesterone and 20β-dihydroprogesterone) are also produced by the hyrax placenta (Mecenero 1998), which may indicate similarities in the steroid metabolic pathway of these two closely related species. Although the elephant placenta does not convert any pregnenolone to progesterone, this conversion does occur in the placentae of other species, amongst others the hyrax (Mecenero 1998), cow (Shemesh, Harel-Markowitz, Gurevich & Shore 1994), baboon (Wango & Mbui 1994), human (Khan-Dawood 1987) and rat (Townsend & Ryan 1970). In some of these species, placental progesterone is essential to maintain pregnancy (Heap 1972).

The amount of pregenolone converted by corpora lutea increased with gestation. The production of the most prominent metabolites, namely 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone, also increased with gestational age. The highest conversion to 11α-hydroxyprogesterone, 17α-hydroxyprogesterone and 20α-dihydroprogesterone occurred during early-, late- and mid-pregnancy respectively. However, these metabolites seem of lesser quantitative importance, especially when compared to the large amounts of 5α-dihydroprogesterone produced by all the corpora lutea. 5α-Pregnane-3α-ol-20-one and 5α-dihydroprogesterone are relatively non-polar and have previously been identified as products of pregnenolone metabolism in African elephant corpora lutea (Hodges *et al.* 1994). The production of these 5α-reduced progestins by the corpora lutea compares well with the concentrations of these progestins measured in African elephant plasma (Hodges *et al.* 1997). The luteal production of 5α-dihydroprogesterone from pregnenolone exceeds the conversion to 5α-pregnane-3α-ol-20-one. Similarly, the circulating concentrations of 5α-dihydroprogesterone were always higher than that of 5α-pregnane-3α-ol-20-one (Hodges *et al.*

1997). Furthermore, the production of progesterone from pregnenolone in corpora lutea is limited and similarly, the circulating concentration of progesterone is also lower than that of 5α-dihydroprogesterone (Chapter 2).

The decrease in the luteal production of the 5α-reduced progestins with gestational age (Hodges *et al.* 1994) reported earlier could not be confirmed in the present study. In fact, the percentage conversion of pregnenolone to 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone increased with gestation. However, in the present study only one corpus luteum from a late-pregnant female was examined. This increase in the production of 5α-dihydroprogesterone is not mirrored in the concentrations of the steroid measured in the plasma (Chapter 2, Fig 5a), where no definite trend in the concentration of the progestin with the gestation period could be illustrated.

There are some similarities in the metabolism of pregnenolone by corpora lutea of the African elephant and hyrax. The close phylogenetic relationship between these two species is based on characteristics such as haemoglobin (Kleinschmidt, Czelusniak, Goodman & Braunitzer 1986) and DNA sequences (Prinsloo 1993). Apart from progesterone, luteal tissue from both these species produced 11α-hydroxyprogesterone and 17α-hydroxyprogesterone (Mecenero 1998). The principal metabolic product of hyrax corpora lutea, namely 17α-hydroxyprogesterone, were not quantitatively as important in the elephant corpus luteum incubations. Furthermore, luteal tissue of the hyrax (Mecenero 1998) did not produce the most prominent metabolites (5α-pregnane-3α-ol-20-one, 5α-dihydroprogesterone, 11α-hydroxyprogesterone and 20α-dihydroprogesterone) of the elephant corpora lutea.

The conversion ability, per unit weight, of elephant luteal tissue exceeded that of the placental tissue. The corpora lutea converted approximately twice the amount of pregnenolone than did the placenta. However, the total metabolism of pregnenolone and therefore the contribution to circulating steroids by the placenta may well exceed that of the corpora lutea, due to its size. The corpora lutea produced mainly the relatively non-polar 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone, with little conversion to the more polar 11α-hydroxyprogesterone, 17α-hydroxyprogesterone and 20α-dihydroprogesterone. The placenta converted the pregnenolone to small percentages of 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone, but more of 11α-hydroxyprogesterone and 17α-hydroxyprogesterone. There seem to be some complimentarity in the abilities of these two tissue types, each probably containing its own suite of enzymes to catalyse these metabolic reactions. The biological significance hereof require further investigation.

Conclusion

The placenta of the African elephant does have the ability to metabolise pregnenolone. It is a less active steroid-producing tissue than the corpus luteum of this species and lacks the ability to produce as large amounts of the two 5α-reduced progestins, namely 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone, as does the corpus luteum. The placenta does therefore not contribute to the production of these two progestins. From both the present study and that of Hodges *et al.* (1994) it is obvious that the corpus luteum of the African elephant is an important, if not the most important, site for the metabolism of pregnenolone. Pregnenolone is a precursor in the biosynthetic pathway and is metabolised in the luteal tissue to the two 5α-reduced progestins which are

considered to be both more representative of ovarian function (*Hodges et al.* 1997) and more biologically active than progesterone (Greyling *et al.* 1997; Greyling *et al.* 1998).

CHAPTER 4

Metabolic conversion of pregnenolone and progesterone by the blood

Introduction

Progesterone is metabolised in the blood of mammals such as foetal (Nancarrow & Seamark 1968) and adult cattle (Choi, Möstl & Bamberg 1989), kid, foetal sheep (Nancarrow & Seamark 1968; Seamark, Nancarrow & Gardiner 1970), lizards (*Tiliqua rugosa*, Bourne 1981), humans, dogs, rabbits, (Van der Molen & Groen 1968) and hyraxes (*Heterohyrax brucei,* Heap, Gombe & Sale 1975; *Procavia capensis*, Makawiti, Osaso & Gombe 1991; Mecenero 1998). Hyrax blood also has the ability to metabolise pregnenolone (Mecenero 1998). Some progesterone metabolism also takes place in human (Scully, Ferguson, Sirrett & Grant 1982) and hyrax leukocytes (Mecenero 1998). The metabolic products are usually reduced forms of progesterone and generally differ from species to species. Due to the close phylogenetic relationship between the elephant and hyrax (Kleinschmidt *et al.* 1986; Shoshani 1986; Prinsloo 1993; Stanhope, Smith, Waddell, Porter, Shivij & Goodman 1996) the metabolic reduction of progesterone by the blood of the elephant may explain the low concentration at which progesterone occur in circulation (see Chapter 2). In the present chapter the ability of elephant blood (whole blood, red and white blood cells and plasma separately) to metabolise pregnenolone and progesterone will be investigated.

Materials and methods

Collection of material

Material was obtained during the population control programme in the Kruger National Park in 1994 and 1995. Blood was collected by exsanguination into heparinised glass tubes and stored on ice for transport to the laboratory.

Incubation trials

All the incubation trials consisted of three treatments. For the first treatment the glass test tubes contained 100µl ^{3}H-pregnenolone (ca. 140 000 dpm), for the second treatment the test tubes contained ^{3}H-pregnenolone and 0.1mM NAD^{+} (Boehringer Mannheim, Mannheim, Germany) and for the third treatment the tubes contained ^{3}H-pregnenolone and enzyme inhibitors (NaF-$HgCl_2$, 10mM, Saarchem, Krugersdorp, S.A.). NAD^{+} is a cofactor for the enzymes which catalyse these reactions (Dorrington 1977; Cheesman 1982). NaF is a general enzyme inhibitor and also specific for hydroxylases, while $HgCl_2$ inhibits dehydrogenases (Heap *et al.* 1975; Inns & Cecchini 1989; Vahdat, Seguin, Whitmore & Johnston 1984). Incubation tubes were prepared prior to the collection of the material each day.

Whole blood (4 ml) was added to each test tube, which were then placed in a shaking water bath at 37°C. One tube of each treatment (^{3}H-pregnenolone, ^{3}H-pregnenolone with cofactor and ^{3}H-pregnenolone with inhibitors) was removed after one, two and three hours respectively, to determine the influence of incubation time on the reaction for each treatment. The incubation mixtures were then centrifuged and the plasma and the cells were stored separately at -20°C. The

red and white blood cells were separated on a Ficoll-gradient (Histopaque 1077, Sigma, St. Louis, U.S.A) according to the method of Böyum (1968). The blood cells were resuspended in Eagle's minimum essential medium. The red blood cell incubations contained 2 ml of this cell suspension, (approximately 5 500 x 10^6 cells, based on published haemotological parameters for African elephants, Brown & White 1980). Each white blood cell incubation contained 100µl of the cell suspension, representing approximately 15 x 10^6 cells (Brown & White 1980). The blood cell incubations were also conducted at 37°C in a shaking water bath for one, two and three hours and afterwards the incubation mixtures were stored at -20°C. For the plasma incubations, blood was centrifuged, the plasma removed and 100µl added to each incubation tube. These were placed in a shaking water bath (37°C) for one, two and three hours (as for the blood incubations) after which they were frozen at -20°C. A control incubation, containing ^{3}H-pregnenolone and Eagle's medium, were incubated alongside the blood experiments as described for the tissue incubations (Chapter 3).

In a separate trial conducted during 1994, 2 ml of whole blood from pregnant females were incubated with ^{3}H-progesterone (ca. 200 000 dpm). The incubation reactions were conducted at 37°C for two hours. As with the ^{3}H-pregnenolone incubations, these incubations were centrifuged and the plasma and cellular compartments stored separately at -20°C. The control incubation for this experiment contained ^{3}H-progesterone and phosphate buffered saline (PBS).

Extraction of steroids

To extract steroids from the whole blood incubations with ^{3}H-pregnenolone, 2 ml of blood cells were lysed with 400µl of 10% Triton X-100 (BDH Chemicals, Poole, U.K.) to release any steroids

which may be in the cells. For the ^{3}H-progesterone incubations with blood, 1 ml of cells were treated with 200µl of 10% Triton X-100. Ethanol (96%, 8 ml and 4 ml for the ^{3}H-pregnenolone and the ^{3}H-progesterone incubations respectively) was added to the lysed cells, the tubes shaken and centrifuged for 10 min. at 2 500 rpm. The supernatant were evaporated under N_2 at 37°C. The pellets were washed twice with an equal volume of 80% ethanol and centrifuged at 2 500 rpm for 10 min. The supernatants were added to the supernatant of the first extraction step and also evaporated. The plasma compartment of the whole blood incubation was also extracted. Ethanol (96%, 4 ml) was added to 1 ml plasma, the tubes shaken and centrifuged for 10 min. at 2 500 rpm. The supernatant was added to the supernatants of the cell compartment and evaporated. The pellet was washed twice with an equal volume of 80% ethanol, centrifuged (10 min., 2 500 rpm) and the supernatants pooled. Scintillation cocktail (4 ml) was added to the final pellet and the radioactivity determined by scintillation counting to determine if any radioactive compounds remained in the pellets. The dried supernatants were reconstituted 1 ml absolute ethanol. An aliquot (200µl) was added to 4 ml scintillation cocktail and the radioactivity determined by scintillation counting to determine the extraction efficiency of radioactive compounds from the incubates. The remainder of the extract was stored at -20°C.

Steroids were extracted from the plasma (750µl), white (350µl) and red blood cell (2.3 ml) incubates with 96% ethanol (3 ml, 1.4 ml and 5 ml respectively). The extraction tubes were shaken for 10 min. and thereafter centrifuged for 10 min. at 2 500 rpm. The pellets were washed twice with an equal volume of 80% ethanol and centrifuged at 2 000 rpm for 10 min. The supernatants from these three steps (96% ethanol and twice 80% ethanol) were pooled and evaporated under a stream

of N_2 at 37°C. The dried extracts were reconstituted in 1 ml absolute ethanol and stored at -20°C. The red blood cell pellets were bleached with H_2O_2 (30%, E.Merck, Darmstadt, Germany) before the radioactivity was determined. Scintillation cocktail (4 ml) was added to the pellets of the white blood cell and plasma extracts and the radioactivity counted to determine if any radioactive compounds remained in the pellets. An aliquot (200µl) of each reconstituted extract was added to 4 ml scintillation cocktail and counted to determine the efficiency of the extraction procedure.

High pressure liquid chromatography (HPLC)

Aliquots (100µl) of extracts were separated as described for tissue extracts (Chapter 3). The mean (± s.e.m.) recovery of radioactive compounds from the HPLC column was 85.2 ± 5.3% (n = 116).

Determination of HPLC peaks

A HPLC fraction was considered a peak if the radioactivity (in dpm) was equal to or exceeded the mean plus four standard deviations of the mean background radioactivity per fraction. Furthermore, the radioactivity in the fraction considered as the peak had to exceed that of the fraction before and after it.

Statistical analysis

All statistical procedures followed Zar (1984). Means are followed by one standard error of the mean (s.e.m.). Mann-Whitney U tests were used to determine differences between different incubation treatments. Significance was taken at the 95% level.

Results

Control incubations

The elution profile of the control incubation for the ^{3}H-pregnenolone incubations (Fig. 8c) show a single peak that eluted in fractions 33-37, the elution position of the ^{3}H-pregnenolone standard (Fig. 8a). The pregnenolone that were added to the control incubations were therefore not metabolised in the absence of blood or the components of blood. Similarly, the elution profile of the ^{3}H-progesterone control incubation (Fig. 11) contained one peak that eluted in the position of the ^{3}H-progesterone standard (Fig. 8b).

Whole blood

The mean extraction efficiency of the procedure for whole blood was 80.9 ± 2.7% (n = 34). The percentage of ^{3}H-pregnenolone that were converted by whole blood ranged between 0 and 52.5%, with the highest mean value (13.3 ± 3.2%) recorded during early-pregnancy. Mean (± s.e.m.) percentage conversions for the three reproductive classes (early-, mid- and late pregnant) are presented in Table 8. The tritiated pregnenolone that were added to the whole blood incubations were converted to eight products, of which progesterone were the most prominant both during early- and late pregnancy (Fig. 12). Other metabolites eluted in the positions of 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 5α-pregnane-3α-ol-20-one, 5α-dihydroprogesterone and Compound I (fractions 54-57). However, all of these conversions were smaller than 5%.

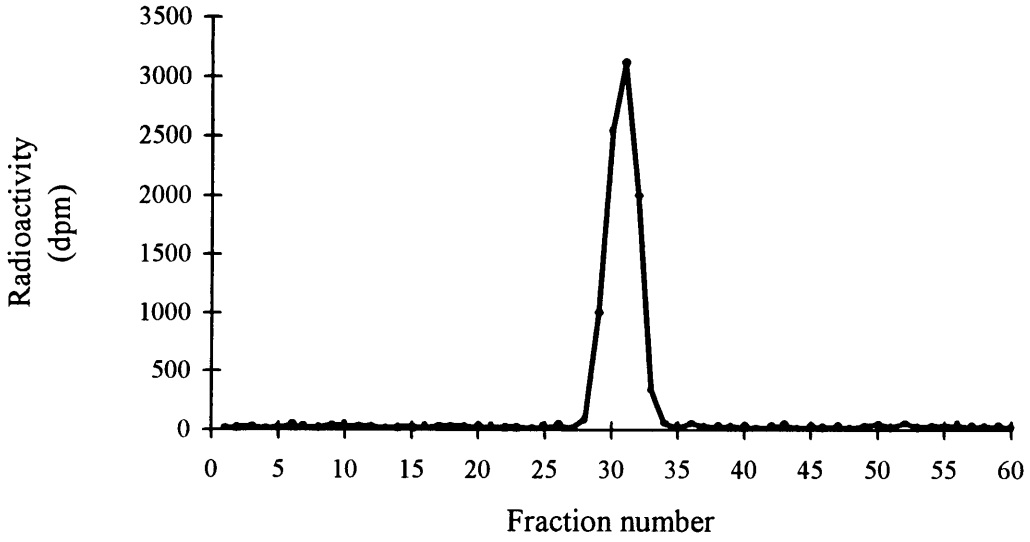

Figure 11 Elution profile of ^{3}H-progesterone that was incubated in the absence of tissue.

Table 8 Percentage (mean ± s.e.m.) conversion of ^{3}H-pregnenolone and production of metabolites by whole blood. The number of incubations for each reproductive class is indicated in parenthesis.

		Metabolites produced (%)							
Reproductive stage	Pregnenolone converted (%)	Progesterone	11α-hydroxy-progesterone	17α-hydroxy-progesterone	20α-dihydro-progesterone	20β-dihydro-progesterone	5α-pregnane-3α-ol-20-one	5α-dihydro-progesterone	I
Early - pregnant (*n*=11)	13.3± 3.2	4.9 ± 1.8	0.8 ± 0.5	1.8 ± 1.3	2.2 ± 1.5	1.1 ± 0.5	0.1 ± 0.1*	0.2 ± 0.2*	0
Mid-pregnant (*n* = 8)	9.1 ± 6.3	0.5 ± 0.3	1.2 ± 0.8	3.1 ± 1.5	1.0 ± 1.0*	2.3 ± 0.8	0.5 ± 0.5*	0	0.4 ± 0.3
Late-pregnant (*n*=10)	11.7 ± 2.5	5.7 ± 1.3	1.6 ± 1.0	2.2 ± 1.3	0.5 ± 0.3	0.5 ± 0.3	0.8 ± 0.6	0.4 ± 0.3	0.6 ± 0.4

*Compound only produced in one incubation.

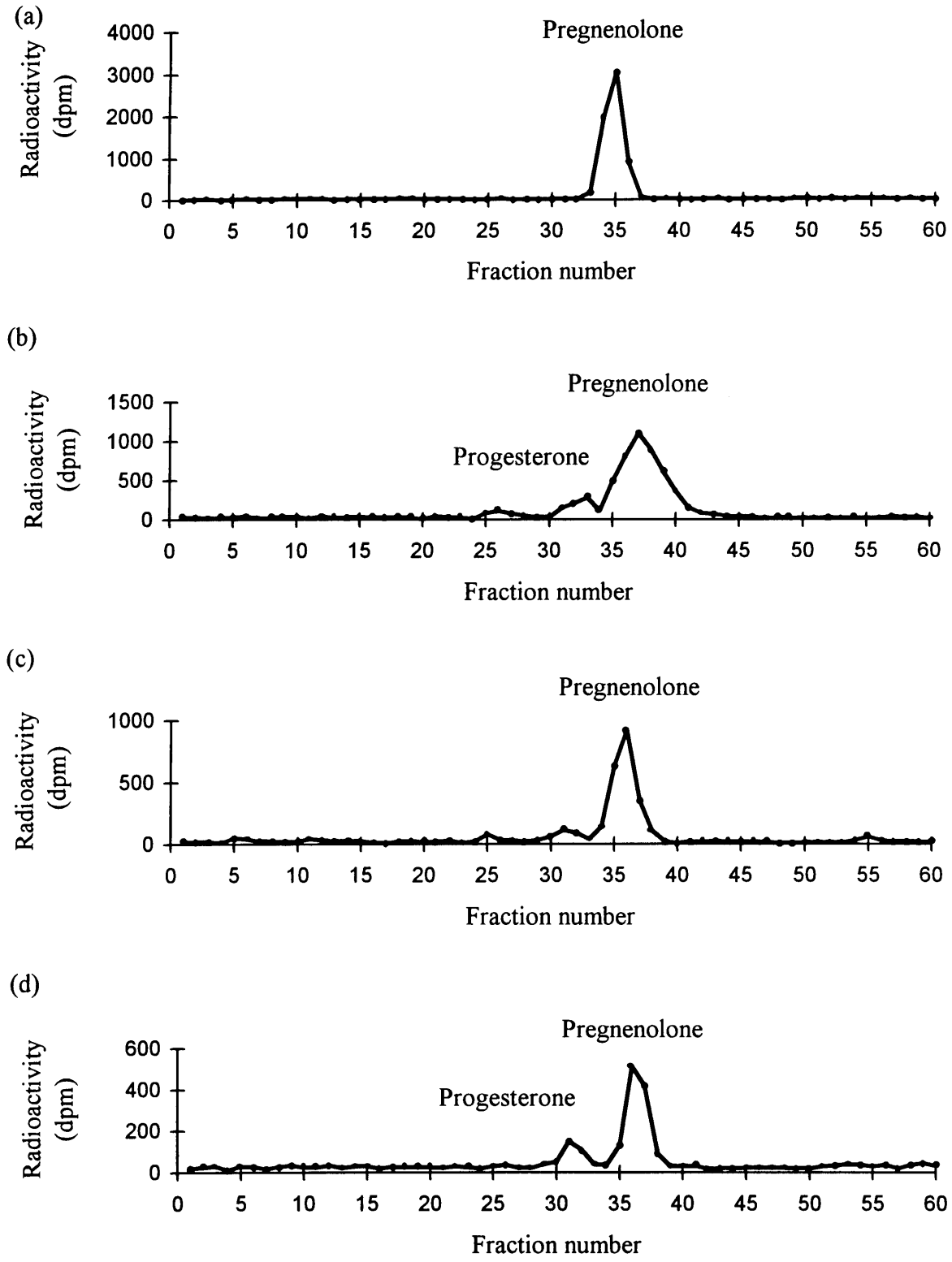

Figure 12 HPLC elution profiles of (a) the control incubation and whole blood incubations with ^{3}H-pregnenolone from an (b) early-, a (c) mid- and a (d) late-pregnant elephant female. The elution positions of pregnenolone and progesterone are indicated.

Tritiated progesterone that were incubated with whole blood were converted to 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone (mean ± s.e.m. percentages presented in Table 9). Elution profiles that were typical for early- and late-pregnant females are presented in Fig. 13. The percentage conversion of ^{3}H-progesterone ranged between 12.0 and 41.2%, with the mean for the late-pregnant category (34.5 ± 0.5%) somewhat higher than that for the early-pregnant females (29.4 ± 8.9%). The conversion of radiolabelled progesterone to 11α-hydroxyprogesterone ranged from 0 to 11.5%, with the mean value for early-pregnant females being slightly higher (9.1 ± 4.8%) than that for late-pregnant females (8.2 ± 0.3%). The conversion of ^{3}H-progesterone to 17α-hydroxyprogesterone ranged from 0 to 10.0%, with the highest mean value (9.9 ± 3.2%) recorded for late-pregnant females. The conversion of progesterone to 20α-hydroxyprogesterone ranged between 0 and 12.3%. The highest conversion (9.9 ± 1.6%) was that by blood from early-pregnant females. The percentage conversion to 20β-dihydroprogesterone ranged from 0 to 7.7%, with the mean for early-pregnant females (6.6 ± 0.2%) being higher than those of late-pregnant females.

Red blood cells

The mean extraction efficiency of radioactive compounds from red blood cells was 85.4 ± 1.9% (n=31). The percentage conversion of tritiated pregnenolone with red blood cells ranged between 0 and 15%, with the highest mean conversion (7.3 ± 1.1%) recorded during early-pregnancy. The pregenolone was converted by red blood cells to progesterone (Fig. 14), 11α-hydroxyprogesterone,

Table 9 Mean (± s.e.m.) percentage conversion of ^{3}H-progesterone and production of metabolites by whole blood. The number of individuals is indicated in parenthesis.

Reproductive stage	Progesterone converted (%)	Metabolites produced (%)					
		11α-hydroxy-progesterone	17α-hydroxy-progesterone	20α-dihydro-progesterone	20β-dihydro-progesterone	5α-pregnane-3α-ol-20-one	5α-dihydro-progesterone
Early-pregnant (*n*=3)	29.4±8.9	9.1 ± 4.8	4.9 ± 3.0	9.9±1.6	6.6±0.2	0	0.2±0.2*
Late-pregnant (*n*=2)	34.5±0.5	8.2 ± 0.3	9.9 ± 3.2	8.9±1.1	3.9±3.9*	1.7±1.7*	0.5±1.4*

*Compound only produced by one animal.

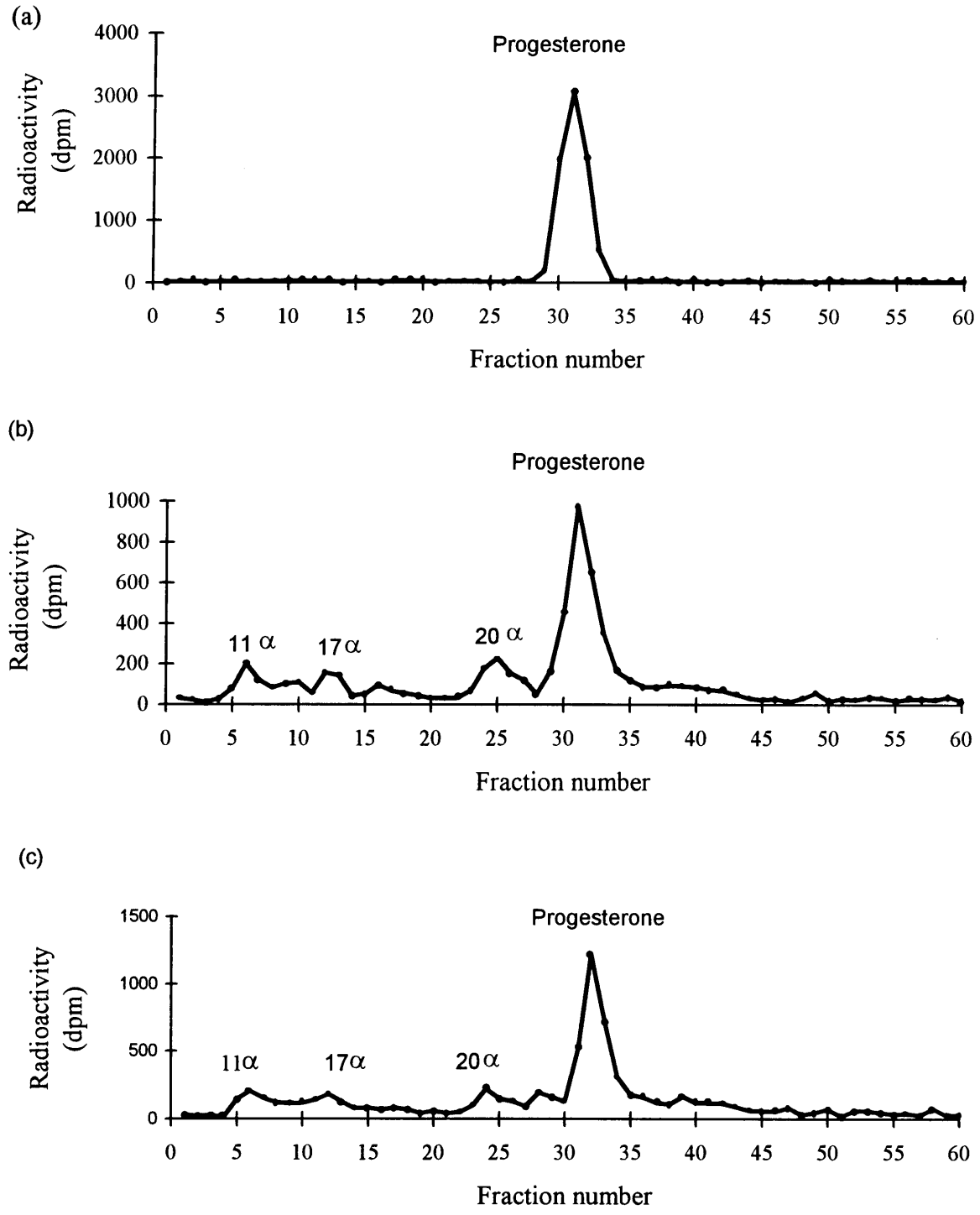

Figure 13 Elution profiles of the (a) control incubation and whole blood incubations from (b) early- and (c) late-pregnant elephants with ^{3}H-progesterone. The elution positions of progesterone, 11α -hydroxyprogesterone (11α), 17 α -hydroxyprogesterone (17α) and 20 α - dihydroprogesterone (20α) are indicated.

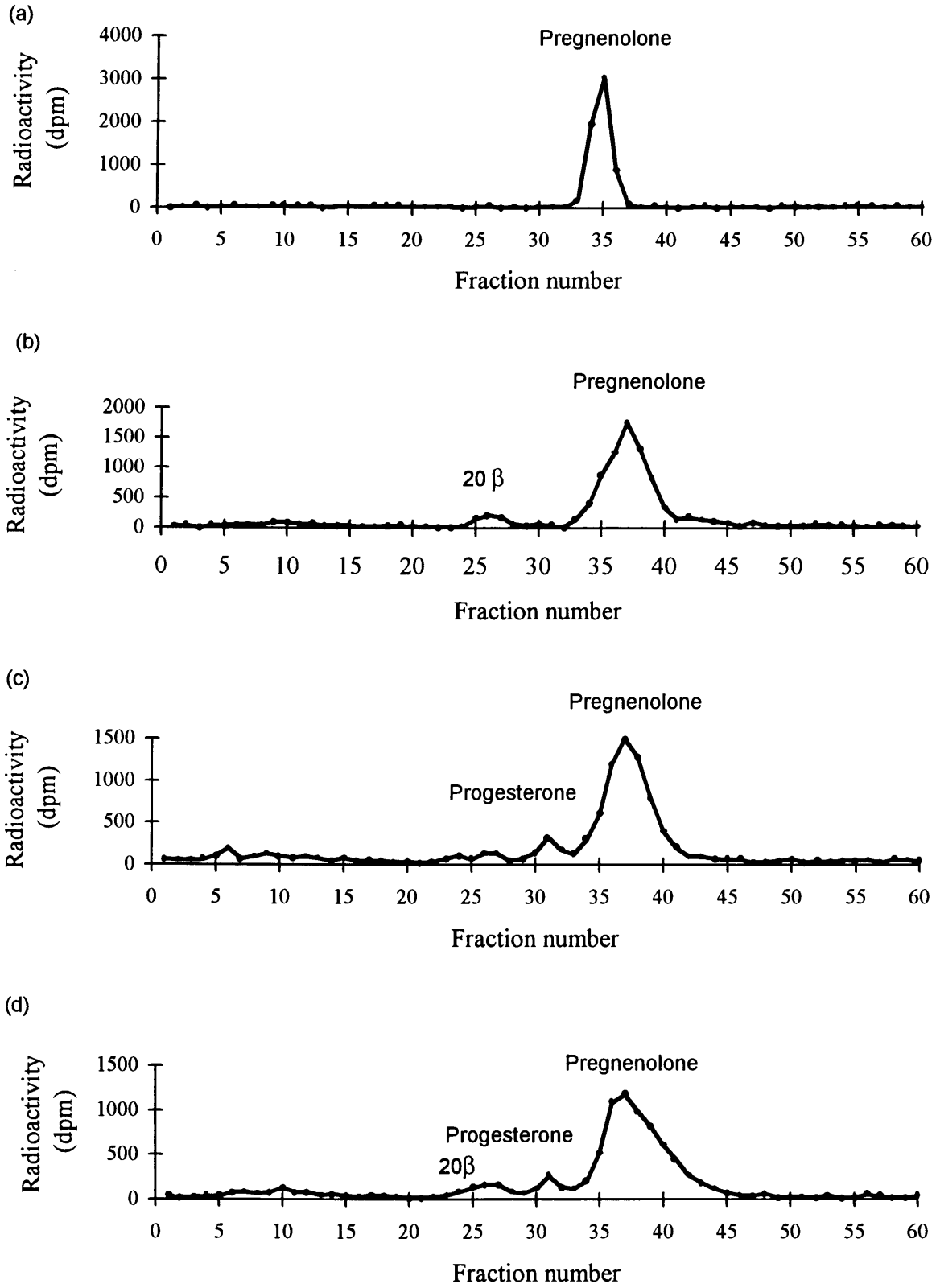

Figure 14 Elution profiles of the (a) control incubation and the red blood cell incubations for (b) eary-, (c) mid- and (d) late-pregnant elephant females. The eultion positions of pregnenolone, progesterone and 20β -dihydroprogesterone (20β) are indicated.

17α-hydroxyprogesterone, 20β-dihydroprogesterone and 5α-pregnane-3α-ol-20-one. The mean percentage conversion of pregnenolone to each metabolite for the three reproductive classes (early-, mid- and late pregnant) are shown in Table 10.

White blood cells

The mean recovery of radioactive compounds from the white blood cell incubations was 82.4 ± 2.3% (n = 28). The conversion of tritiated pregnenolone by the white blood cells ranged from 0 to 13.5%, with the highest mean value (6.0 ± 1.9%) recorded during early-pregnancy. Pregnenolone was converted to progesterone (Fig. 15), 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone and 20β-dihydroprogesterone. The mean (± s.e.m.) percentage conversion of ^{3}H-pregnenolone and the metabolites produced are presented in Table 11. The conversion of pregnenolone to progesterone varied from 0 to 5.5%, with the mean for late-pregnant females the highest (2.9 ± 0.8%). The percentage conversion to 11α-hydroxyprogesterone ranged from 0 to 6.8% and the most (3.0 ± 0.8%) thereof was produced by white blood cells from late-pregnant females.

Plasma

The mean (± s.e.m.) efficiency for the extraction of radiolabelled compounds from plasma was 79.9 ± 2.3% (n = 29). The percentage of ^{3}H-pregnenolone that were converted ranged from 0 to 10.6%. The amount of pregnenolone converted increased with gestation period. These conversions, as well as the metabolites produced in the incubation reactions are presented in Table 12. The products of the plasma reactions were progesterone (Fig. 16) and small amounts of 11α-hydroxyprogesterone,

Table 10 Mean (± s.e.m.) percentage conversion of ^{3}H-pregnenolone and production of metabolites by red blood cells. The number of incubations for each reproductive class is indicated in parenthesis.

Reproductive stage	Pregnenolone converted (%)	Metabolites produced (%)				
		Progesterone	11α-hydroxy-progesterone	17α-hydroxy-progesterone	20β-dihydro-progesterone	5α-pregnane-3α-ol-20-one
Early-pregnant (n = 11)	7.3 ± 1.1	0.9 ± 0.3	1.2 ± 0.4	0.7 ± 0.2	4.1 ± 1.0	0.1 ± 0.1*
Mid-pregnant (n = 10)	6.9 ± 1.4	1.3 ± 0.2	1.5 ± 1.0	0.8 ±0.4	3.3 ± 0.8	0
Late-pregnant (n = 10)	6.8 ± 1.3	1.0 ± 0.3	1.7 ± 1.2	0.6 ± 0.3	4.5 ± 1.1	0.1 ± 0.1*

*Compound only produced in one incubation.

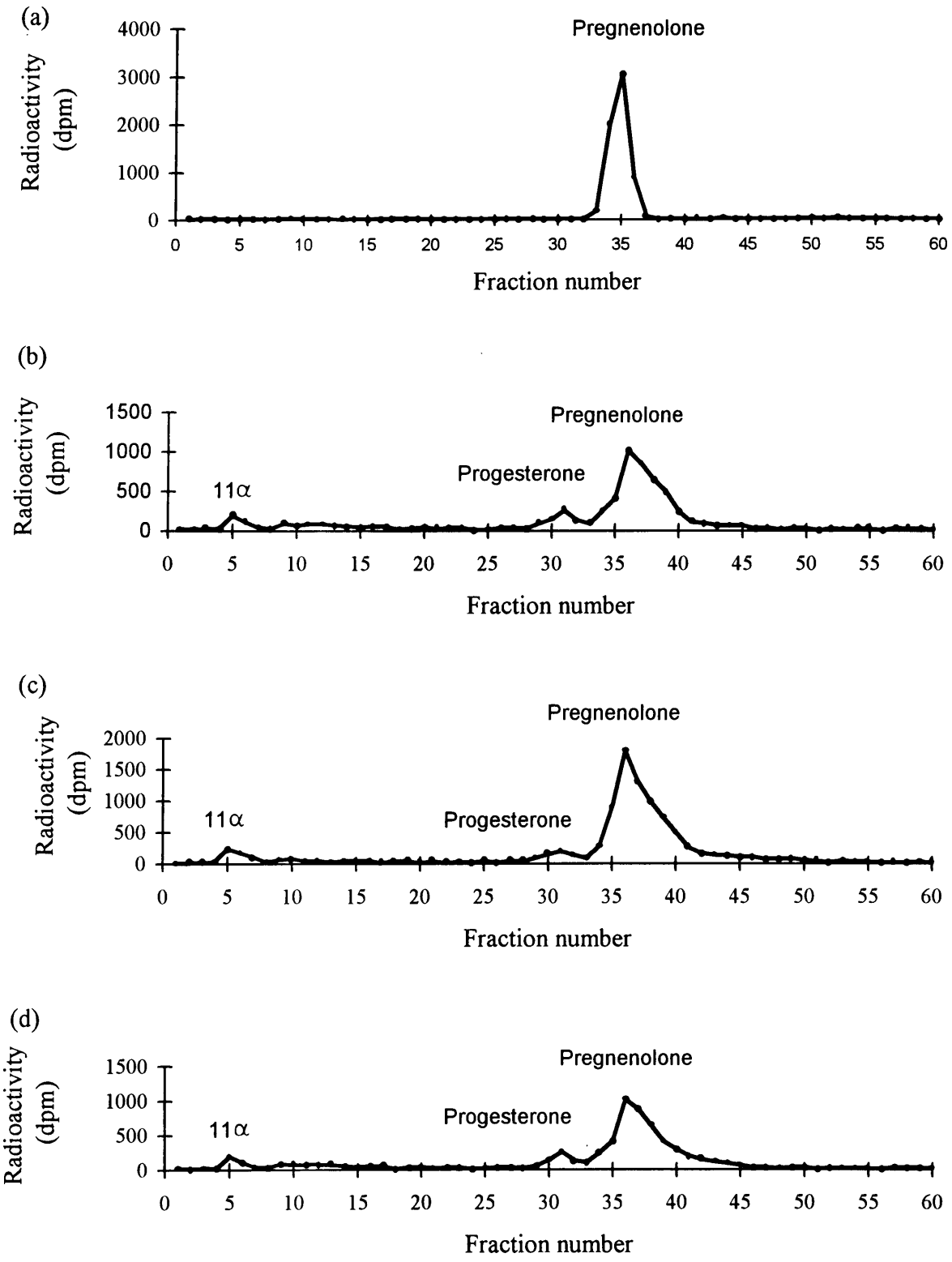

Figure 15 Elution profiles of (a) the control incubation and white blood cell incubations from (b) early-, (c) mid- and (d) late-pregnant elephant females. The elution positons of pregnenolone, progesterone and 11α -hydroxyprogesterene (11α) are indicated.

Table 11 Percentage (mean ± s.e.m.) conversion of ^{3}H-pregnenolone and production of metabolites by white blood cells. The number of incubations for each reproductive class is indicated in parenthesis.

Reproductive stage	Pregnenolone converted (%)	Metabolites produced (%)				
		Progesterone	11α-hydroxy-progesterone	17α-hydroxy-progesterone	20α-dihydro-progesterone	20β-dihydro-progesterone
Early-pregnant (*n*=11)	6.0 ± 1.9	1.3 ± 0.3	2.0 ± 1.5	1.7 ± 0.5	0	0.4 ± 0.2
Mid-pregnant (*n*=10)	3.1 ± 0.9	0.9 ± 0.4	2.7 ± 1.1	0.4 ± 0.2	0	0.2 ± 0.1
Late-pregnant (*n*=7)	5.6 ± 1.3	2.9 ± 0.8	3.0 ± 0.8	2.1 ± 0.6	0.1 ± 0.1*	0.3 ± 0.2

*Compound only produced in one incubation.

Table 12 Percentage (mean ± s.e.m.) conversion of ^{3}H-pregnenolone and production of metabolites by elephant plasma. The number of incubations for each reproductive class is indicated in parenthesis.

Reproductive stage	Pregnenolone converted (%)	Metabolites produced (%)						
		Progesterone	11α-hydroxy-progesterone	17α-hydroxy-progesterone	20α-dihydro-progesterone	20β-dihydro-progesterone	5α-pregnane-3α-ol-20-one	5α-dihydro-progesterone
Early-pregnant (*n*=11)	3.5 ± 0.6	1.3 ± 0.6	1.4 ± 1.5	0.5 ± 0.3	0.2 ± 0.1	0.7 ± 0.2	0	0
Mid-pregnant (*n*=10)	4.9 ± 0.8	5.6 ± 1.1	0.9 ± 0.3	1.6 ± 0.5	0	0	0.1 ± 0.1*	0.3 ± 0.3
Late-pregnant (*n*=8)	7.3 ± 0.8	4.8 ± 1.4	0.8 ± 0.5	0.8 ± 0.4	0	0.4 ± 0.3	1.2 ± 1.2*	0

*Compound only produced in one incubation.

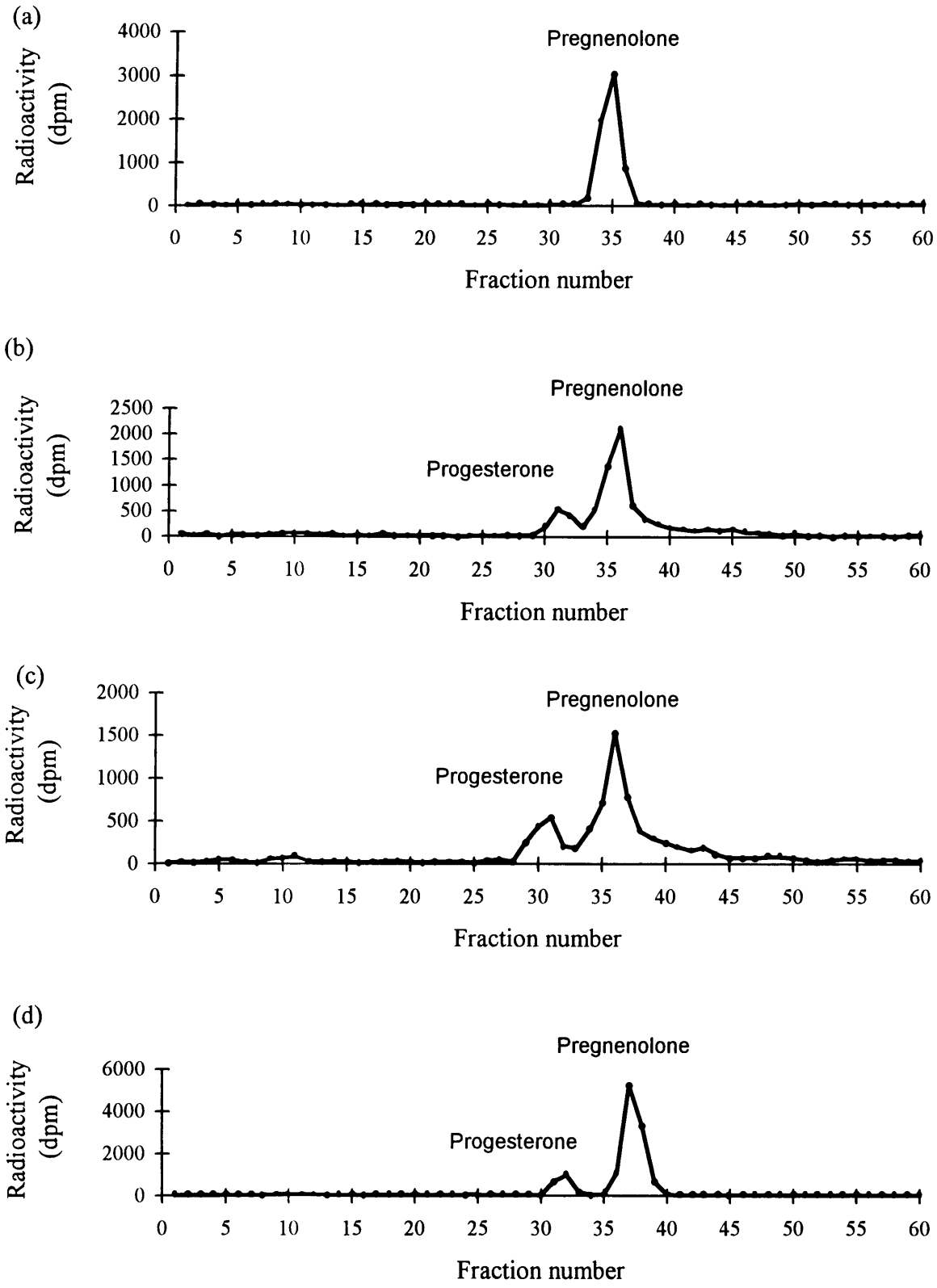

Figure 16 Elution profiles of (a) the control incubation and plasma incubations for (b) early-, (c) mid- and (d) late-pregnant elephant females. The elution positions of pregnenolone and progesterone are indicated.

17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone, 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone. The highest mean conversion (5.6 ± 1.1%) of ^{3}H-pregnenolone to progesterone was during mid-pregnancy, while the conversion for all three reproductive classes ranged between 0 and 11.5%.

Influence of time on the reaction

The mean (± s.e.m.) conversion of pregnenolone by whole blood, red blood cells, white blood cells are presented in Table 13. In the incubations with whole blood, the most pregnenolone (11.7 ± 2.7%) were converted during the first hour. There were no significant differences between the amount of pregnenolone converted after one, two and three hours. For red blood cells, there were no significant differences ($p > 0.05$) between the mean (s.e.m.) percentage pregnenolone converted after one (5.6 ± 0.6%), two (6.0 ± 0.9%) and three hours (6.5 ± 1.5%). However, in the white blood cell incubations, the pregnenolone converted after three hours (7.0 ± 1.0%) was significantly higher ($Z(U=16) = -2.2$, $p < 0.05$) than that converted after one hour (4.6 ± 1.6%). Similarly, the pregnenolone converted after three hours (5.8 ± 0.7%) by the plasma incubations were significantly higher ($Z(U=15) = -2.3$, $p < 0.05$) than the pregnenolone converted after the first hour (4.0 ± 1.0%) of incubation.

Influence of cofactor on the reaction

The influence of the enzyme cofactor (NAD^{+}) in the incubation mixture on the percentage of pregnenolone (mean ± s.e.m.) converted by the different blood components is shown in Table 14.

Table 13 The influence of incubation time on the conversion of ^{3}H-pregnenolone by elephant blood. The values are means (± s.e.m.) of the percentage of pregnenolone converted (n = 9).

Blood compartment incubated	Pregnenolone converted (%)		
	60 min.	120 min.	180 min.
Blood	11.7 ± 2.7	8.2 ± 1.0	9.3 ± 4.0
Red blood cells	5.6 ± 0.6	6.0 ± 0.9	6.5 ± 1.5
White blood cells	4.6 ± 1.6	5.3 ± 1.0	7.0 ± 1.0
Plasma	4.0 ± 1.0	4.0 ± 0.9	5.8 ± 0.7

Table 14 The influence of the presence of cofactor (NAD^+) in the incubation mixture on the convertion of pregnenenolone by elephant blood. The values are means ± s.e.m. of the percentage pregnenolone converted ($n = 9$).

Blood compartment incubated	Pregnenolone converted (%)	
	^{3}H-pregnenolone	^{3}H-pregnenolone & NAD^+
Blood	15.1 ± 3.1	13.9 ± 4.2
Red blood cells	7.3 ± 0.6	7.5 ± 1.2
White blood cells	5.6 ± 1.1	3.3 ± 0.7
Plasma	5.1 ± 0.9	5.1 ± 1.1

For all the blood components incubated, there were no significant differences ($p > 0.05$) between the incubations with and those without the cofactor.

Influence of enzyme inhibitor on the reaction

The incubations of all the blood components that contained the enzyme inhibitors (NaF-$HgCl_2$) converted less pregnenolone than those incubations that did not contain the inhibitors. A summary of these results for different blood components in the presence and absence of inhibitors is presented in Table 15. Although the amount of pregnenolone converted by the incubations that contained the inhibitors was lower, it was only in the incubations with red blood cells that this difference was statistically different ($Z(U=11) = -2.6, p < 0.05$).

Discussion

The conversion of pregnenolone by the blood of the elephant peaked during the early part of the gestation period, but the differences between animals from different stages of pregnancy were small. Progesterone is the most prominent metabolite produced, with the highest percentage conversion ($5.7 \pm 1.3\%$) during late-pregnancy. Although circulating progesterone levels are not elevated during late-pregnancy (see Chapter 2), progesterone may be required for the production of other progestins (Heap & Flint 1979). The conversion of progesterone was also much higher than that of pregnenolone. These findings suggests that progesterone may be an intermediate for the production of other progestins in the biosynthetic pathway. The metabolites produced in both (^{3}H-pregnenolone and ^{3}H-progesterone) incubation experiments were similar, but the percentages that

Table 15 The influence of the addition of enzyme inhibitors ($NaF\text{-}HgCl_2$) to the incubation mixture on the convertion of pregnenolone by elephant blood. Each value is the mean ± s.e.m. percentage of pregnenolone converted ($n = 9$).

Blood compartment incubated	Pregnenolone converted (%)	
	^{3}H-pregnenolone	^{3}H-pregnenolone & $NaF\text{-}HgCl_2$
Blood	13.5 ± 3.1	6.8 ± 3.0
Red blood cells	7.3 ± 0.6	3.3 ± 1.0
White blood cells	5.6 ± 1.1	4.8 ± 1.8
Plasma	5.1 ± 0.9	4.1 ± 0.6

were converted in the incubations with progesterone were higher than that produced in the pregnenolone incubations.

Some aspects of the progestin metabolism in elephant blood can be compared to that of hyrax blood (Mecenero 1998). In both species progesterone is more readily metabolised than pregnenolone. 11α-Hydroxyprogesterone and 17α-hydroxyprogesterone were produced in small quantities by both elephant and hyrax blood. However, different to the hyrax, elephant blood did not produce 5α-dihydroprogesterone and 5α-pregnane-3α-ol-20-one (Mecenero 1998). Bovine blood also metabolised pregnenolone and progesterone to 17α-hydroxyprogesterone (Choi *et al.* 1989). Human and sheep blood produced 20α-hydroxyprogesterone by progesterone (Van der Molen & Groen 1968), while 5α-dihydroprogesterone was produced in *in vivo* experiments with rabbit blood (Verma & Laumas 1981).

During the present study the ability of isolated red blood cells to convert pregnenolone was limited and not affected by stage of gestation. Progesterone, 11α-hydroxyprogesterone, 17α-hydroxyprogesterone and 20β-dihydroprogesterone were the major products of the conversion, with the latter the most prominent metabolite. However, elephant red blood cells converted more pregnenolone that those of the hyrax (Mecenero 1998).

In the elephant white blood cells only converted a small amount of pregnenolone. As with the blood and red blood cells, progesterone was one of the most important metabolites. Some of the other metabolites were only produced during one stage of gestation or in one incubation. This differs

from the significant conversion of pregnenolone and progesterone to the 5α-reduced progestins by hyrax white blood cells (Mecenero 1998).

In incubations with all the components of blood, progesterone was the principal metabolite and it seems as if this production of progesterone detected in the whole blood incubations could be due to the cumulative production by plasma, red and white blood cells. However, high concentrations of progesterone is not required during the gestation period (Plotka *et al.* 1975; Mc Neilly *et al.* 1983; De Villiers *et al.* 1989; Chapter 2).

In the elephant blood plays a minor role in the metabolism of progestins. The corpora lutea is the most important tissue for the production of progestins, especially the 5α-reduced progestins, 11α-hydroxyprogesterone and 17α-hydroxyprogesterone were produced in all the tissues examined.

The influence of time on the progress of the reactions was inconclusive. Most reactions showed an increase in the metabolism of pregnenolone with time. The influence of NAD^+ as a cofactor was less than expected. In only approximately half of the incubations analysed, did the incubations with the cofactor convert a higher percentage of pregnenolone, and in those cases, the increases in the percentage conversion were not high. One of the other cofactors, for example $NADP^+$ or NADPH, may be more specific. The enzyme inhibitors, proved to be more effective in decreasing the pregnenolone metabolised in most of the incubations. Even though the percentage of pregnenolone converted were lower in the incubations that contained the inhibitors, the reactions were never

completely prevented from taking place. This may however be achieved by using a higher concentration of NaF-$HgCl_2$.

Conclusion

The blood of the African elephant, and its components, has a low potential to metabolise both pregnenolone and progesterone. The main metabolite produced in the incubations with whole blood, red blood cells, white blood cells and plasma was progesterone. The progesterone that was produced in the whole blood is a result of the combined effects of the metabolism in the cells and plasma. From the incubations of ^{3}H-progesterone with whole blood is was evident that the progesterone (and more of it) could be metabolised further. However, the specific compartment of blood responsible for these conversions is not yet known, and should be the subject of further investigation. Different to the phylogenetically related hyrax, elephant blood does not directly contribute to the synthesis of 5α-dihydroprogesterone and/or 5α-pregnane-3α-ol-20-one.

CHAPTER 5

Synthesis

The present study supports previous reports of circulating concentrations of progesterone (Plotka *et al.* 1975; McNeilly *et al.* 1983; Brannian *et al.* 1988; Plotka *et al.* 1988; De Villiers *et al.* 1989; Schwarzenberger *et al.* 1996; Hodges *et al.* 1997), 5α-dihydroprogesterone (Hodges *et al.* 1997) and oestradiol-17β (Plotka *et al.* 1975; Hodges *et al.* 1983; Hodges *et al.* 1987; Brannian *et al.* 1988; Plotka *et al.* 1988; Kapustin *et al.* 1996) in pregnant and non-pregnant elephant cows. The very low (<64.7pg/ml) or undetectable levels of oestradiol-17β suggest that the measurement of another oestrogen or oestrogen conjugate could be more informative. Concentrations of progesterone and 5α-dihydroprogesterone were significantly higher in pregnant than in non-pregnant females, but the ranges in the concentrations of these progestins in pregnant and non-pregnant females overlapped to such an extent that the plasma concentrations of neither progesterone nor 5α-dihydroprogesterone can be used to detect or confirm pregnancy. Concentrations of 5α-dihydroprogesterone were higher than those of progesterone in pregnant females. This supports the suggestion that plasma concentrations of this 5α-reduced progestin provide a better reflection of ovarian function than concentrations of progesterone (Hodges *et al.* 1997). 5α-Dihydroprogesterone has also been suggested to be the more biologically active progestin in the African elephant (Hodges *et al.* 1994; Schwarzenberger *et al.* 1996; Wasser *et al.* 1996; Hodges *et al.* 1997). Biological activity was confirmed by the relatively high binding affinity of the uterine progesterone receptor for this progestin (Greyling *et al.* 1997). However, this binding

to the receptor should not be treated in isolation, as there are additional conditions that have to be met for a metabolite to be of significant biological importance (Higgins & Gehring 1978).

Although plasma progesterone concentrations were similar during all stages of gestation (early-, mid- and late-gestation), the production of progesterone from pregnenolone by the corpus luteum (Hodges *et al.* 1994, Chapter 3) and blood (Chapter 4) was the highest during early-pregnancy. At mid-pregnancy progesterone production in both the blood and luteal tissue (Hodges *et al.* 1994; Chapter 3) decreased. This is not the same as the conclusions that can be drawn from the circulating levels of progesterone. The role of progesterone, as indicated by its binding to uterine receptors (Greyling *et al.* 1997), cannot be ignored. The situation for 5α-dihydroprogesterone is similar, with no significant differences in plasma concentrations between early-, mid- and late-pregnant females, but an increase in the percentage of 5α-dihydroprogesterone that was produced from pregnenolone by the corpus luteum with the gestation period.

The significance of the more polar 11α-hydroxyprogesterone and 17α-dydroxyprogesterone produced by the placenta and blood is not clear. These metabolites are probably derivatives of progesterone, as are 5α-dihydroprogesterone and 5α-pregnane-3α-ol-20-one, which would to some extent justify the conversion of pregnenolone to progesterone by the corpus luteum (Hodges *et al.* 1994; Chapter 3) and blood. The synthesis of hormones is a dynamic process. Different sites of hormone production seem to increase and decrease in activity in order to compliment each other at different stages of gestation, so that the endocrinological demands can be met throughout the gestation period. Progestin metabolism by whole blood, red and white blood cells is was important

during the early stages of the gestation period. As the pregnancy progressed to the mid-pregnant stage, the contribution of the placenta and possibly the corpora lutea and plasma became more significant. During late-pregnancy the corpora lutea, plasma, whole blood, white blood cells and possibly red blood cells were metabolising the most progestins.

SUMMARY

In an attempt to find methods to control the size of elephant populations in confined areas through the manipulation of reproductive output, the present study focused on some of the fundamental questions of the reproductive process in female African elephants. The use of reproductive technologies would offer an alternative to the culling programmes which have been used until very recently (1995). The objectives of the study were to determine whether the circulating concentrations of progesterone, 5α-dihydroprogesterone and oestradiol-17β could be used to detect pregnancy in the female African elephant and if these concentrations in pregnant females and foetuses followed specific patterns during the gestation period. The relationship, if any, between the different hormones and between pregnant females and their foetuses were examined. Furthermore, the potential of elephant placenta, corpora lutea and blood to metabolise pregnenolone, a precursor for steroid hormone synthesis, was examined. The ability of these tissues to produce 5α-dihydroprogesterone and 5α-pregnane-3α-ol-20-one, and thereby contribute towards the circulating levels of these biologically significant progestins, was determined.

Circulating concentrations of progesterone, 5α-dihydroprogesterone and oestradiol-17β were measured in plasma from pregnant and non-pregnant females and foetuses. Although there were significant differences between the concentrations of progesterone and 5α-dihydroprogesterone in pregnant and non-pregnant females, the ranges of the two groups overlapped. The plasma concentrations of oestradiol-17β were low regardless of the reproductive status of the animal. There were no obvious trends in the circulating concentrations of these three reproductive steroids during gestation. No relationship between foetal and maternal plasma concentrations could be

illustrated. It is concluded that the circulating concentrations of progesterone, 5α-dihydroprogesterone or oestradiol-17β can not be used to diagnose pregnancy in the female African elephant.

The ability of the placenta, corpus luteum and blood (and its separate components) to synthesise steroid hormones from a radiolabelled precursor (pregnenolone), was determined. The placenta converted some (<38%) pregnenolone to 11α-hydroxyprogesterone and 17α-hydroxyprogesterone. The corpora lutea converted most of the pregnenolone (up to 82.7%), with 5α-pregnane-3α-ol-20-one and 5α-dihydroprogesterone as principal products. Whole blood converted up to 11.7% of the pregnenolone, with progesterone as the main compound produced. Whole blood could metabolise higher percentages of progesterone (41.2%). The products were similar to those produced in the pregnenolone incubations. Progesterone itself was also the principal conversion product of red blood cells, white blood cells and plasma that were incubated with pregnenolone. Red blood cells, white blood cells and plasma converted less pregnenolone than whole blood and the activity in the whole blood is probably due to the cumulative contributions of the blood cells and plasma. The percentage conversion of pregnenolone was similar throughout the gestation period (early-, mid- and late pregnancy). Some of the incubation reactions were influenced by time, such that higher percentages of pregnenolone was converted and/or higher percentages of products formed in the longer incubations. In contrast to this, the enzyme inhibitors (NaF-$HgCl_2$) reduced the conversion of pregnenolone and/or the production of metabolites in most of the incubations. Of all the tissues examined, only the corpus luteum produced 5α-dihydroprogesterone and 5α-pregnane-3α-ol-20-one from ^{3}H-pregnenolone. However, the blood produced some progesterone from which these

5α-reduced progestins were probably synthesised. The influence of the enzyme inhibitors indicates that these steroidogenic reactions can only occur in the presence of enzymes.

SAMEVATTING

In 'n poging om metodes te vind waardeur die grootte van olifantbevolkings in bewaringsgebiede, deur die manipulasie van voortplanting, beheer kan word, het hierdie projeck gefokus op sommige fundamentele vrae in verband met die voortplantingsproses in die Afrika-olifantkoei. Die doelstellings van die projek was as volg: om te bepaal of die konsentrasies van progesteroon, 5α-dihidroprogesteroon en oestradiol-17β in die plasma gebruik kan word om dragtigheid in die olifantkoei te bevestig; om te bepaal of hierdie konsentrasies in dragtige koeie, sowel as in fetusse, spesifieke patrone deur die dragtigheidstydperk volg; om te bepaal of daar 'n onderlinge verband tussen die vlakke van hierdie drie hormone en tussen die konsentrasies gemeet in dragtige koeie en hul fetusse bestaan; om te bepaal of die plasenta, korpus luteum en bloed pregnenaloon kan metaboliseer; om te bepaal of die plasenta, korpus luteum en bloed 5α-dihidroprogesteroon en/of 5α-pregnaan-3α-ol-20-oon kan vervaardig en sodoende 'n bydrae tot die konsentrasie van hierdie twee belangrike progestiene in die plasma van die olifantkoei kan maak.

Die konsentrasies van progesteroon, 5α-dihidroprogesteroon en oestradiol-17β is in die plasma van dragtige en nie-dragtige koeie en van fetusse gemeet. Alhoewel daar betekenisvolle verskille in die konsentrasies van progesteroon en 5α-dihidroprogesteroon tussen dragtige en nie-dragtige diere was, het die waardes oorvleuel. Die konsentrasie van oestradiol-17β was laag in die plasma van al die diere. Daar is geen duidelike neigings in die konsentrasie van hierdie hormone gedurende die draagtydperk bespeur nie. Geen verband tussen fetale en materne plasma-konsentrasies kon aangedui word nie. Die konsentrasie van progesteroon, van 5α-dihidroprogesteroon of van oestradiol-17β kan dus nie gebruik word om te vas te stel of 'n olifantkoei dragtig is nie.

Die vermoë van die plasenta, corpus luteum en bloed (en die komponente daarvan) om steroïedhormone vanaf radioaktiewe pregnenaloon te vervaardig, is bepaal. Die plasenta het klein hoeveelhede (<38%) van die pregnenaloon omgeskakel na 11α-hidroksieprogesteroon en 17α-hidroksieprogesteroon. Die corpus luteum kon die meeste (tot 82.7%) van die ^{3}H-pregnenaloon omskakel en die belangrikste produkte van die reaksie was 5α-pregnaan-3α-ol-20-oon en 5α-dihidroprogesteroon. Bloed het tot 11.7% van die pregnenaloon waarmee dit geïnkubeer was, omgeskakel na progesteroon. Heelbloed kon ook hoër persentasies van ^{3}H-progesteroon metaboliseer. Die produkte van hierdie reaksies was dieselfde as dié van die pregnenaloon-inkubasies. Progesteroon was die belangrikste produk van die reaksies met rooibloedselle, witbloedselle en plasma, al was die persentasie omskakeling laer as dié van heelbloed. Die produksie van progesteroon wat in heelbloed gemeet is, is heel waarskynlik die kumulatiewe effek van die bloedselle en plasma. Die omvang van die omskakeling van pregnenaloon was dieselfde vir al drie stadia van dragtigheid. Sommige van die reaksies is deur die tydsduur van die inkubasie beïnvloed sodat 'n hoër persentasie van pregnenaloon omgeskakel is en/of produkte vervaardig is in die langer inkubasies. Die teenwoordigheid van ensieme is deur die suksesvolle inhibisie van die reaksie deur NaF-$HgCl_2$ aangetoon. Van al die weefsels wat ondersoek is, was net die corpus luteum in staat om 5α-dihidroprogesteroon en 5α-pregnaan-3α-ol-20-oon te vervaardig. Die bloed het wel progesteroon vervaardig, wat heel waarskynlik nodig is vir die vervaardiging van die 5α-progestiene. Die invloed van die inhibeerders het aangeton dat hierdie reaksies slegs kan plaasvind in die teenwoordigheid van ensieme en dat die aktiwiteit van hierdie ensieme beheer kan word.

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